USES OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY [HPLC] IN THE ANALYSIS OF DRUGS SUCH AS CAPTOPRIL CANDESARTAN PROPRANOLOL TERAZOSIN VERAPAMIL AND IMPURITY PROFILE OF CITALOPRAM METAXALONE AND ONDANSETRON

THESIS

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BY

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2007

DECLARATION

I, hereby declare that the work embodied in this thesis has been carried out by me under the guidance of Dr. S.N Shrivastava, Head of Department (Chemistry), Bipin Bihari P.G College, Bundelkhand University, Jhansi U.P. No part of this thesis had been submitted by me anywhere for obtaining any degree or diploma.

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This is to certify that the thesis entitled "Uses of high performance Liquid Chromatography [HPLC] in analysis of Drugs such as Captopril, Candesertan, Propranolol, Terazosin, Verapamil and impurity profile of Citalopram, Metaxalone and Ondansetron" submitted to B.U. Jhansi U.P. India for the award of the degree of Doctor of Philosophy in Chemistry is a record of bonafide research work carried out by Smt. Monika Jain under my guidance and supervision. The work embodied in this thesis or a part here of has not been submitted for the award of any other degree or diploma. She remain present and work in my guidance for 200 days.

S.N. Shrivastava

Dr.S.N.Srivastava
Supervisor

Dedication

A small token of love and respect to
my parents(shri P.L Jain and smt Aradhana Jain)
and my husand(Anish) our daughters(Adavita & Archita)

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LIST OF PUBLICATIONS.

1. A stability indicating assay method for captopril tablets by high performance liquid chromatography for stability studies. Analytical Chemistry: An Indian Journal Volume and issue 3(2-3), 2006. web publication: 11/10/2006.

2. A stability-indicating assay method for Verapamil tablets by high performance Liquid chromatography for stability studies . Analytical Chemistry: An Indian Journal Volume and issue 4(4-6), 2007. web publication: 21/12/2006.

3. A stability-indicating assay method for candesartan tablets by high performance liquid chromatography for stability studies . Analytical Chemistry: An Indian Journal Volume and issue 5(1-6),2007. web publication: 5/5/2007.

4. A stability-indicating assay method for Propranolol tablets by high performance liquid chromatography for stability studies . Analytical Chemistry: An Indian Journal. Volume and issue 6(1),2007. web publication: 22/7/2007.

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LIST OF ABBREVIATIONS

Abbreviations	Description
AAS	Atomic Absorption Spectrophotometry
CDER	Centre for Drug Evaluation and Research
CE	Capillary Electrophoresis
FIA	Flow Injection Analysis
GC	Gas Chromatography
GC-MS	Gas Chromatography - Mass Spectrophotometry
GLP	Good Laboratories Practice
HETP	Height Equivalent to Theoretical Plate
HPLC	High Performance Liquid Chromatography
HPL TC	High Performance Thin Layer Chromatography
IC	Ion Chromatography
ICH	International Conference On Harmonisation
LC-MS	Liquid Chromatography - Mass Spectrophotometry
LOD	Limit of Detection
LOQ	Limit of Quantification
MEKC	Micellar Electrokinetic Chromatography
NMR	Nuclear Magnetic Spectrophotometry
PDA	Photo Diode Array
RSD	Relative Standard Deviation
RT	Retention Time
SFC	Supercritical Fluid Chromatography
SOP	Standard Operating Procedure
SS	System Suitability
SWV	Square-Wave Voltametry
TLC	Thin Layer Chromatography
USFDA	United States Food and Drug Administration
USP	United States Pharmacopoeia
UV	Ultraviolet
WHO	World Health Organisation

LIST OF INSTRUMENTS

Sr. No.	Instrument	Model	Company
1.	**Shimadzu HPLC System**		Shimadzu Corporation, Japan
	Solvent delivery pump	LC-10ATVP	
	UV-Vis detector	SPD-10 AVP	
	Photo diode array detector	SPD-M10A	
	Autosampler	SIL-10AVP	
	System controller	SCL-10 AVP	
	Column oven	CTO -10 ASVP	
	Class VP software	Ver. 6.0	
2.	**UV-Vis Spectrophotometer**	UV 160 A	
3.	**Weighing Balance**	AE 240	Mettler USA
4.	**pH meter**	Digital pH meter	Elico Ltd Hyderabad
5.	**Ultrasonic Bath**	Ultrasonic water bath	Toshniwal Brothers, Mumbai
6.	**Water Bath**	water bath	Toshniwal Brothers, Mumbai
7.	**Centrifuge**	Acm-67891-C	Acmas Technocracy LTD. Delhi
8.	**Vaccum Pump**	Vaccum pump	Toshniwal Brothers, Mumbai
9.	**HPLC Grade Water Preparing Unit**	Milli-Q system	Millipore Corporation, USA
10.	**Glassware**		
a	1.0 ml graduated pipette	A grade-certified	Hirschmann Laborgerate Germany
b	5.0 ml graduated pipette	A grade-certified	
c	10.0 ml graduated pipette	A grade-certified	
d	5.0 ml bulb pipette	A grade-certified	
e	10.0 ml volumetric flask	A grade-certified	
f	25.0 ml volumetric flask	A grade-certified	
g	50.0 ml volumetric flask	A grade-certified	
h	100.0 ml volumetric flask	A grade-certified	

LIST OF CHEMICALS

Sr. No.	Name of Chemical	Grade	Make
1.	Acetonitrile	HPLC	E. Merck (India) Ltd., India
2.	Diammonium hydrogen ortho Phosphate	AR	S. d. Fine-Chem Ltd., India
3.	Disodium hydrogen ortho Phosphate	AR	S. d. Fine-Chem Ltd., India
4.	Formic acid	AR	S. d. Fine-Chem Ltd., India
5.	Hydrochloric acid	AR	S. d. Fine-Chern Ltd., India
6.	Hydrogen peroxide	AR	E. Merck (India) Ltd., India
7.	Methanol	HPLC	Qualigens Fine Chemicals, India
8.	Methane Sulphonic acid	AR	E. Merck (India) Ltd., India
9.	Phosphoric acid	AR	S. d. Fine-Chem Ltd., India
10.	Potassium dihydrogen ortho Phosphate	AR	S. d. Fine-Chem Ltd., India
11.	Sodium hydroxide	AR	Qualigens Fine Chemicals, India
12.	Tetrahydrofuran	HPLC	E. Merck (India) Ltd., India
13.	Triethylamine	AR	Qualigens Fine Chemicals, India
14.	Water	HPLC	Milli-Q filtered water

LIST OF DRUGS

Sr. No.	Drug	Company
1.	Candesartan	Brand name-Ipsita, Bal Pharma, Bangalore
2.	Captopril	Brand name-Aceten, Wockhardt, Mumbai
3.	Propranolol	Brand name-Loten, Aurochem lab., Mumbai
4.	Terazosin	Brand name-HYTRIN, Abbott lab India, Mumbai
5.	Verapamil	Brand name-Calaptin, Nicholas Piramal LTD. Mumbai
6.	Citalopram	SPIL, Mumbai
7.	Metaxalone	SPIL, Mumbai
8.	Ondansetron	Sodhana lab LTD, Hyderabad

CHAPTER - 1

INTRODUCTION

Analytical chemistry is a branch of chemistry dealing with separation and analysis of chemical substances. Analytical chemistry[1] includes both qualitative and quantitative analysis. Qualitative analysis is concerned with "What is present" and quantitative analysis with "How much is present". Analytical chemistry finds extensive applications in the analysis of organic compounds, pharmaceuticals, biochemicals, body fluids, soils and many other types of substances and in pollution control. Pharmaceutical analysis[2-10] is an important area of application of analytical chemistry; specially in industries.

1.1 IMPORTANCE OF PHARMACEUTICAL ANALYSIS

Healthcare is the promotion of well being and prevention of ill health. This can be achieved by proper nutrition, potable water, adequate shelter, clothing, clean air and sanitary facilities. Prevention and curing of ill health can be done by use of proper drugs.Drug is broadly defined as any chemical agent that affects living processes[11]. As the drug involves life, its quality becomes vital. There is no "second" quality in drugs. Pharmaceutical research has assumed great dimensions in terms of finding new molecules effective in countering the diseases, as well as it should be safe and without any side effects.

During the development of new potential drugs, detailed chemical studies must be made of raw materials, intermediates and final formulations. These studies must identify the types and levels of impurities, degradation products, degradation rate etc.

The information resulting from these studies is used for the rates of degradation of the drug and its formulation under a variety of conditions. This information is needed to define conditions for storage and handling of drugs, that will assure potency throughout the expected shelf life of the product. Stability studies are especially demanding of analytical precision and accuracy because changes of a few percent over a period of five years are considered significant and must be accurately quantitated. In order to detect such small changes, analytical methods must be very precise and free from interference from degradation product and/or excipients used in making formulations. Over the past two decades, various leading international regulatory bodies such as USFOA, WHO, British Pharmacopoeial Commission etc., have taken very serious view about the quality of bulk drugs and formulations. Center for Drug Evaluation and Research (CDER) in USA is one of such leading agency, which is issuing timely guidance to industry as regard to various Good Manufacturing Practices norms, formulation design, bioequivalence studies, monitoring of stability of products and its storage conditions, validation of analytical methods etc. USFDA regulations so far are supposed to be the most stringent in these regards and they focus not only on the physical and chemical attribute of the product, but also on the bioequivalence of new generic products to the innovator products. Due to these stringent regulatory and ethical requirements, it becomes equally important to monitor the quality of these drugs with respect to identity, potency and purity at the time of release and various stages throughout the shelf life. Therefore, a set of analytical procedures developed to control the quality of products must include both qualitative and quantitative methods in order to assure the identity and purity of the products. These are achieved using various analytical techniques.

1.2 ANALYTICAL TECHNIQUES

Various analytical techniques are used in pharmaceutical analysis as shown in Table 1.1. Generally, quantitative analytical procedures are classified into two categories as, chemical methods and instrumental methods.

1.2.1 Chemical Methods[12]

The quantitative execution of chemical reaction is the basis of chemical methods. In gravimetric analysis, the substance being determined is converted into an insoluble precipitate, which is then collected and weighed, or in special case of electrogravimetry, electrolysis is carried out and the material deposited on one of the electrodes is weighed.

In titrimetric analysis, the substance to be determined is allowed to react with an appropriate reagent added as a standard solution, and the volume of solution needed for complete reaction is determined. The common types of reactions used in titrimetry are neutralization (acid-base) reactions; complex forming reactions; precipitation reactions and oxidation-reduction reactions. It is very widely applicable approach in quantitative analysis[13-15]. A titration is feasible when

(a) the titration reaction is rapid compared to the speed of titration

(b) it's equilibrium constant is large enough to give a sharp "break" at the end point and

(c) a method of end point detection is available.

Table 1.1 : Analytical techniques used in pharmaceutical analysis

TECHNIQUES	APPLICATIONS
Chemical Methods	
Gravimetry	Semi quantitative assay
Titrimetry	Assay of bulk drugs
Thermal Methods	
Differential Scanning Calorimetry	Polymorphism, Drug excipient interaction, melting point of coloured compounds
Differential Thermal Analysis	Moisture determination
Electroanalytical Methods	
Coulometry	Moisture determination
	Studying the chirality of molecule
Spectrophotometry Methods	
X-ray Diffraction	Identification and polymorphism
UV-Vis	Identification and assay
Infrared	Identification
NMR	Structure elucidation
AAS	Estimation of different elements
Mass	Molecular weight determination
Fluorometry	Identification and assay
Flow Injection	Assay
Polarimetry	Studying the chirality of molecule
Separation Techniques	
Paper / Thin Layer Chromatography	Identification and semi quantitative estimation
HPTLC	Separation and estimation of different compounds
Gas Chromatography	Separation and estimation of thermally stable volatile compounds
High Performance Liquid Chromatography(HPLC)	Separation and estimation of wide range of compounds

1.2.2 Instrumental Methods[16-18]

These methods are dependent either upon the measurement of an electrical property or determination of the extent to which radiation is absorbed or emitted; and require the use of a suitable instrument, e.g., spectrophotometer, spectrofluorometer etc., and in consequences such methods are referred to as 'instrumental methods'. Instrumental methods are usually much faster than purely chemical methods. They are normally applicable at concentrations far too small to be amenable to determination by classical chemical methods and are more selective than chemical methods. In most cases a computer can be interfaced to the instrument so that absorption curves, chromatograms, titration curves, etc., can be plotted automatically, and in fact, by the incorporation of appropriate servo-mechanisms, the whole analytical process may be completely automated.

Instrumental methods of analysis can be further classified into

a. Spectrophotometric Methods
b. Flow injection Methods
c. Fluorimetric Methods
d. High Performance Thin Layer Chromatographic Methods
e. Gas Chromatographic Methods
f. High Performance Liquid Chromatographic Methods

These instrumental methods have been discussed below in detail:

a) Spectrophotometric Methods

Spectrophotometry is the most widely used technique in the pharmaceutical industry. Among the various physicochemical methods, UV-Vis spectrophotometry is the most commonly used analytical tool[19-22]. As compared to other physicochemical methods UV- Vis Spectrophotometry has several advantages:

(i) High sensitivity incase of suitable chromophore.

(ii) No special qualification is required for handling instrument.

(iii) It can be coupled with suitable separation techniques (HPLC, TLC, etc) and

(iv) It is useful for quantitative examination of multicomponent mixture.

The main disadvantages are:

(i) It cannot be generally applied for trace analysis and

(ii) The methods are often not selective.

The analysis of organic compounds via functional groups represents a powerful tool for the analyst. All drug molecules possess one or more functional groups that can be analysed in some fashion. However, when a functional group method is employed, it is specific only for that particular group. A drawback of these methods is that degradation products of the drug may also possess the functional group and hence will be analysed along with the parent molecule. In this case, the assay may not be stability indicating. If, on the other hand, the functional group being analysed is destroyed during the degradation of the drug, the assay may not be stability indicating.

Spectrophotometric methods involve the absorption of light radiation of specific wavelength which is characteristic of the particular molecule. The quantitation is based on Lambert-Beer's law, which states that the amount of radiation absorbed by the sample at particular wavelength is proportional to the concentration of the light absorbing substance in that sample. Determinations by using this nature of a compound are generally made in the ultraviolet region. The samples that do not absorb significant amount of light can be treated with a suitable reagent to convert

them into new species that absorb light intensely.

The reagent should react selectively and stoichiometrically with the substance to be estimated. Conditions for optimum colour formation like pH of reaction medium, solvent composition, reagent concentration, order or reagent addition etc., must be determined. The coloured product formed should be stable for appropriate time and should have acceptable molar absorptivity. In addition, factors like availability, cost, stability and colour of the reagent also play an important role in selection of reagent. The approaches available to convert the sample into an absorbing species are oxidation reaction, oxidative coupling reaction, diazotization and coupling, charge transfer complexation, ion-pair complexation and complexation with metal ions.

b) Flow Injection Analysis

It is an interesting development that an old technique sometimes achieves new importance when it is modified with the incorporation of modern advances in instrumentation. A variety of approaches have been introduced during recent years to the automation of wet chemical analysis. However, because of the development of physical methods (e.g. spectroscopy and chromatography) over the same period, the application of wet chemical analysis has received little attention. In situations where physical methods are inapplicable, automated wet chemical methods have been actively pursued. This is particularly true of process control and clinical analysis where frequent tests are required on a large number of similar samples. The combination of specificity and the low cost of chemical methods with speed and reproducibility of instrumentation have led to extensive use of automated wet chemical analysis in these fields. Flow injection analysis[23] (FIA) is one such approach where sample is injected to a continuous flow of reagent solution. The sample and

reagent are allowed to mix in a coil and passed through a detector. The detector response is recorded for quantification. Conceptually, FIA can be viewed as HPLC without column. In HPLC, column provides the specificity while in FIA, a combination of chemistry and the detector may provide the required specificity. Most of the theory of chromatography, which is based on chemical engineering transport of theory, developed to describe mass transport and dispersion in a narrow tube can be applied to FIA.

In recent years, FIA has proved to be relatively inexpensive and useful technique with practical applications in the areas of clinical chemistry[24-26] and other fields[27-28]. The features of FIA that make it attractive for pharmaceutical analysis[29-30] are the short start up time, simplicity of instrumentation and high sampling rate. The technique is suitable for batch type analysis where a few to hundreds of samples are to be analysed. A sample injected into a carrier stream flowing through a narrow bore section of tube exists as a well defined plug and subsequently disperses and mixes with the carrier stream. Coiling the tube can reduce dispersion in a FIA system. The peak shape will be sharp for limited dispersion.

The type of dispersion desired is dependent on the requirements of the analytical method and is obtained by varying the instrumental parameters such as reaction coil length, internal diameter of the coil, flow rate, reagent concentration etc. At high flow rates, dispersion increases, residence time decreases and reagent consumption increases. Optimization of parameters is required to maximize the sensitivity and sample throughput, which minimizes reagent and sample consumption.

c) Fluorimetric analysis

Fluorescence analysis[31-35] is an analytical method closely related to spectrophotometry. A molecule gets excited from its ground electronic state to an excited electronic state by absorbing energy in the form of visible or ultraviolet light. Many molecules are capable of emitting this energy as radiation, thus returning to the ground state. The emitted radiation is called 'fluorescence' which is directly proportional to the concentration of absorbing molecule. A necessary condition for fluorescence spectrophotometry is a strong absorption by the molecule. Aromatic, heterocyclic and highly conjugated structures, all of which cause intense absorption, are therefore apt to impart fluorescent properties to a molecule.

d) High Performance Thin Layer Chromatography[36-38]

High performance thin layer chromatography (HPTLC) is a highly instrumental technique carried out on efficient, fine particle layers, capable of producing fast, high resolution separations and quantitative results with accuracy and precision rivaling those of gas chromatography (GC) and high performance liquid chromatography (HPLC). It is highly selective and flexible because of great variety of layers that are available. It has proven to be as sensitive as column liquid chromatography in many analyses, and solvent usage per sample can be extremely low, leading to fewer disposal problems. The prominent advantages of this technique include:

(i) Possibilities of separating and analysing upto 25 samples and standards simultaneously on a single plate leading to high throughput, low cost analyses, and the ability to construct calibration curves from standards chromatographed under the same conditions as the samples.

(ii) Analysing the samples with minimal cleanup, including those containing

components that remain sorbed to the origin of the layer or suspended microparticles.

Analysing a sample by use of multiple separation steps and static post chromatographic detection procedures with various universal and specific visualization reagents, which is possible because all the sample components are stored on the layer without the chance of loss.

Today, HPTLC has enjoyed widespread popularity for the separation of lipids and aromatics, sugars catecholamines, steroid harmones, carboxylic acids, sulphonic acids, nucleotides, phenols, racemic mixtures of amino acids and pharmaceuticals.

e) Gas Chromatography

Gas chromatography[39-45] is one of the most extensively used separation technique in which the separation is accomplished by partitioning a solute between a mobile gas phase and stationary phase, either liquid or solid. It provides a quick way of determining the components in a mixture, including the presence of impurities, and in many cases, prima facie evidence of the identity of compound. The chief requirement is some degree of stability at the temperature necessary to maintain the substance in a gas state. Today, GC is a powerful tool for the separation and quantitation of complex-organic, metal-organic, biochemical and pharmaceutical systems.

f) High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) [46-52] is increasingly becoming the premier choice for analysing pharmaceutical raw materials and their formulations, according to regulatory agencies and compendia. The advantages of HPLC include simplicity, accuracy, precision, versatility and most importantly the selectivity, especially to distinguish between constituents of a multicomponent system. The

ability of HPLC to separate degradation products makes it very useful in the analysis of pharmaceutical formulations.

Liquid chromatography is basically a separation technique wherein various types of mixtures can be separated into their constituent components. The purpose of the separation may either be to quantitate each of the separated compounds in order to evaluate performance characteristics or to prepare large amounts of the samples for further use.

Over the past few years, HPLC has been one of the fastest growing analytical techniques. Unlike in GC analysis where sample must be volatile, the main criterion in HPLC is that the sample must be soluble. More than 80% of known organic compounds can be analysed by HPLC. A typical system consists of a solvent delivery pump, which is required to force the mobile phase through the column at a constant flow rate. Some kind of injection device is used to introduce a solution of the sample into the solvent stream. The sample and mobile phase travel through the heart of the system - the column. The sample component gets separated and elutes from the column and enters into the eye of the HPLC system - the detector. Various types of detectors are available,

the choice of which depends on the particular compounds to be monitored and sensitivity levels required. A recording device can be used to record the detector signal as a function of time called the chromatogram.

1.3 GOOD LABORATORY PRACTICE IN ANALYTICAL LABORATORY

As the quality of products is becoming more and more important, the need for the good laboratory practices is being increasingly recognized in many laboratories. It is not only important from the regulatory point of view, but also from the ethical point.

The proper GLP program helps in improving the overall productivity by ensuring precise and accurate results. It also improves the confidence and morale of chemists working in analytical laboratory. Some of the most important activities, which are associated with good laboratory practices, are discussed here.

1.3.1 Calibration and Validation of Analytical Equipments

Results obtained from a properly validated method can go haywire; if the instrument used for the analysis is not properly calibrated and validated. Some of the common errors, which can arise from the use of a non validated / calibrated instrument are shown in Table 1.2.

Table 1.2 : Effects of instrumental parameters on results

Sr. No.	Parameter	Effect
01	Wavelength accuracy	Error in assay using E1 %
02	Pump flow	Error in retention time
03	Temperature accuracy of oven	Error in loss on drying values
04	Temperature accuracy of thermometer	Error in melting point and boiling point determination
05	Calibration of burette	Error in titrimetric analysis

The instrument used for the analysis should be satisfactorily validated to avoid instrumental errors.

1.3.2 Validation of Analytical Methods

Results obtained from all the techniques discussed above may not yield useful information unless enough amount of validation is carried out before employing a newly developed method for regular analysis. Various regulatory guidelines are available on the protocol to be followed for an exhaustive method validation. International Conference on Harmonization (ICH) guidelines is the most widely

followed amongst them. Various common parameters, which need validation, are listed in the Table 1.3 and discussed.

Table 1.3 : Analytical parameters for validation

Sr. No.	Parameter
a	System precision
b	Stability of analytical solutions
c	Specificity
d	Linearity of response
e	Method precision
f	Accuracy (Recovery)
g	Robustness
h	Limit of quantification
i	Limit of detection

a) System Precision (System Suitability Parameters)

It is the ability of the system to perform accurately. This includes all the hardware and data acquisition and is considered as one unit. It is established by six consecutive measurements of response (area counts I absorbance) of the standard solution or system suitability solution (if specified in the method) and calculate the relative standard deviation (RSD). The relative standard deviation of 2.0 %(assay) and 5.0 %(impurity profile) indicates good system precision. In case of HPLC, RT for analyte , resolution factor with respect to any other active component or degradation product (if any), tailing factor and number of theoretical plates of main peak should be established and determined on each day the analysis is being carried out.

b) Stability of Analytical Solutions

With the development of various automated instruments, it is possible to measure the

response of solutions unattended for many hours. But basic requirement of this is that the analytical solution has to be stable throughout the measurement period. It is established by analyzing standard and sample solutions at different time intervals and comparing it with a freshly prepared standard solution of similar concentration. Typically, solution is considered to be stable for the time till its purity is within ± 2.0 % (assay) and ± 5.0 % (impurity profile)of its original purity level.

c) Specificity

Specificity of a method is its ability to measure accurately and specifically the analyte in presence of components that may be present in the sample matrix. Specificity for an assay ensures that the signal measured comes from the substance of interest, and there is no interference from excipients and/or degradation products and/or impurities. In case of HPLC analysis of formulation/bulk drug, it is established by:

1. Proving no interference from any of the excipients used in the formulation. This is achieved by injecting a placebo preparation. There should be no peak in the chromatogram from the placebo at the retention time of the analyte peak.
2. Proving no interference from any of the degradation products in the analyte peak. This is ensured by carrying out forced degradation studies on:

i. Active raw material or working reference standard: This gives an idea about various probable degradation products of the active raw material.

ii. Placebo: This will give evidence for identification of degradation products from placebo, if any.

iii. Formulation blend

Generally following conditions are used for the forced degradation:

,Acid degradation (2.0 N HCl)+ 60 min on boiling water bath

Alkaline degradation (2.0 N NaOH)+60 min on boiling water bath

Oxidative degradation by H_2O_2 (3 %)+60 min on boiling water bath

Thermal degradation 60 min on boiling water bath

Photolytic degradation in Sun light for 4 hours

pH of the solution was adjusted to 7.0 if necessary, and dilute the sample as per the procedure. Analyze the treated product by HPLC using a diode array detector and record the peak purity of analyte peak. The method is said to be specific if the peak purity of analyte peak is 100 (total peak purity) and 0.99(3 point peak purity) for assay and impurity profile analysis respectively.

d) Linearity of Response

It is a measure of the method's ability to obtain results, which are directly proportional to the concentration of the analyte within a given range. The range of the procedure is an expression of the lowest and highest levels of the analyte that the method can determine with reasonable accuracy and precision. This evaluation process will demonstrate the nature of the calibration curve. It is established by measuring the response of standard solutions of at least 5 different known concentrations equivalent to about 50%, 75%, 100%, 125% and 150% of the standard concentration used in assay or impurity profile.

The result of the linearity test is analysed by a linear regression method by plotting a calibration curve following the general equation of a straight line ($y = mx + c$) using concentration as abscissa and response as ordinate and represented in terms of coefficient of regression (r). Typical acceptance level for r is not less than 0.99.

e) Method Precision

Precision of the method is a measure of reproducibility of results. It is determined by

analyzing six replicate sample preparations as per the method and calculating assay or quantifying impurity of each sample. It is expressed in terms of relative standard deviation. Typical acceptance level for RSD is not more that 2.0 % (assay) and 5.0 % (impurity profile).

f) Accuracy (Recovery)

Accuracy of an analytical method is the closeness of test results obtained by that method to the true value. It is a measure of exactness of the analytical method. It is established by preparing recovery samples by spiking drug in placebo (if available) or in pre analysed sample at three different levels (i.e. 80%, 100% and 120% of label claim) and analyzing them. For impurity profile at 5 different levels(i.e.70%, 85%, 100%, 115% and 130% of standard solution) Whenever it is not possible to spike the active pharmaceutical ingredient by weighing, a stock solution of the same should be prepared in suitable solvent and added to the placebo. A typical acceptance level is 98.0 -102.0%(assay) and 95.0 – 105%(impurity profile) recovery.

g) Robustness

Robustness of the method is a measure of reproducibility of results when same analysis is carried our under a variety of normal test conditions.It is established by carrying out analysis of the same sample by deliberately varying certain experimental condition such as analyst, pH of the solution, composition of mobile phase, temperature in chromatographic analysis and Instrument change etc. Many such parameters can be varied judiciously depending upon the complexity anticipated in carrying out that particular analysis and ensuring that a practical deviation from the originally specified condition does not significantly alter the results obtained.

h) Limit of Quantification

It is the lowest concentration of analyte that can be determined quantitatively with acceptable accuracy and precision using the recommended procedure of analysis. The method based on the residual standard deviation of a regression line and slope was adopted. To determine the LOQ, a specific calibration curve was constructed using samples containing the analytes in the range of LOQ. This can be confirmed practically by measuring the response of the standard solution and its subsequent dilutions. A typical acceptance level is RSD not more than 5.0% for triplicate injections.

i) Limit of Detection

It is the lowest concentration of substance in a sample that can be detected, but not necessarily quantitated. Usually this is achieved by measuring the signal to noise ratio for the analyte at different concentration. The concentration giving a signal to noise ratio of 3 is considered as a limit of detection or by visual identification.

1.3.3 Validation of Analyst

It is relatively new concept in validation, which ensures that the analyst uses the method and machine judiciously in order to get the most accurate and reproducible results. This generally involves making an analyst familiar with the machine, methods and systems by providing him with a set of properly documented standard operating procedures and testing instructions.

1.3.4 Importance of Documentation

All the experiments carried out in the laboratory needs to be documented with great care and detail, as without this, it is impossible to have a systematic functioning of the

laboratory and keep a track of product quality and understand the trend. The documentation normally required in a laboratory may be divided into the following broad categories:

1. General Standard Operating Procedures (SOP'S). related to receipt of material and equipments and entry of personnel.
2. SOP'S related to the use of various sophisticated equipments.
3. Different standard testing procedures.
4. Record of installation and maintenance of various equipments.
5. Record of different samples and their analytical reports.

1.4 STABILITY STUDIES[53-55]

A drug is a substance having pharmacological effects and intendend for therapeautic uses. Some drugs are suspectibile to chemical degradations under various conditions owning to their fragelitiy of the molecular structure and other drug substance undergo physical degradation changes leading to change in their physical state. chemical degradation as well as physical degradation may change their pharmaceutical effects, resulting in altered efficacy therapeautic as well as toxicological consequences, because pharmaceutical are used on therapeautically based on their efficacy and safety, they should be stable and maintained their quality until their time of usage or until their expiry date. The quality should be maintained under various conditions that pharmaceuticals encounter, during production, transportation and storage in warehouses, pharmacy shops and homes. Therefore understanding the ways which alters the stability of pharmaceuticals is very important.

The stability guidelines make certain requirements on basic stability of drugs[56].First of

all it is necessary to develop stability indicating assay. This is define in line 111 of 1987 guidelines of as “Quantitate analytical method that are based on the characteristic structural, chemical or biological properties of each active ingredients of the drug products and that will distinguish each active ingredients from its degradation products so that the active ingredient product can be correctly measured.” The 1999 ICH guidelines states,

Analytical test procedures should be fully validated and the assay should be stability indicating. The need or the extent of replication will depend on the results of validation studies.(194-196).

The focus may instead be on assuring the specificity of the assay, identified degradants as indicator of the extent of degradation via particular mechanisim.(386-389).

This means that assay must be capable of detecting quantitative the amount of parent drug present, and to identify ...to some degree quantify the degradation products. In developing stability indicating assay methodology, it is customary to deliberately decompose the drug in solution, so as to challenge the assay and insure its capabilities of separating the parent drug from degradation products.

The FDA guidelines on drug product stability provides clear guidelines on stability protocol, test parameters, acceptance criteria and procedures, test intervals(long term, accelerated and intermediate),container storage(upright, inverted and horizontal) and test storage conditions(25°C/60 % RH, 40°C/60 % RH, 30°C/60 % RH) .For product packed in semipermeable containers the storage conditions are (40°C/15 % RH, 25°C/40 % RH, 30°C/40 % RH).The FDA requires stability data from minimum three batches to evaluate batch to batch variability and also requires the

expiry date on the basis of long term stability data.

All materials observed during stability studies of the drug product should be summarized.[57] This summary should be based on degradation pathways and laboratories studies. If any impurities are excluded because they are not degradation products, the rationale for this exclusion should be presented. If identification is not possible, then the reason for it should be explained. This should be substantiated with extensive laboratories studies. Generally degradation products below the indicated level do not need to be identified unless they are suspected of unusually potent or toxic. Specification limits for degradation products or drug – excipient interaction should be based on following combinations.

1) Stability studies

2) Knowledge of degradation pathways

3) Product of development studies

4) Laboratories studies

Limits should be set for specified and unspecified degradation products as well as total degradation products. Batch to batch variations in impurities should be explained.

1.5 SCOPE OF PRESENT WORK

There are various analytical techniques, which have been used to monitor the quality of pharmaceutical products. The concept of quality in the pharmaceutical industry has been changing continuously. The new quality concept says that the quality has to be built not only in the final product but also in the system. Quality control and quality

assurance[58] functions today have greater significance than ever and are no more considered being a liability on to a company's expenditure plan. More and more companies are recognizing the importance of these two functions and are trying to distinguish them from each other. The role of quality control is to study the various attributes of the final product, whereas the role of quality assurance comes into picture from very beginning of the product manufacturing and extends till the distribution and storage phase. A properly designed quality control and assurance plan can save the company from unwanted rejection due to inconsistent quality.

With the increasing importance of quality functions, various regulatory authorities are becoming more and more demanding. It is not only essential to ensure the presence of required amount of the drug in the formulation throughout its shelf life, but also to control the extent of related substances, degradation products and various other contaminants. This is because some of the degradation products may be toxic, may have undesirable adverse pharmacological effect, may alter the bioavailability of the drug. Acceptable limit of such impurities is very less, to the extent of 0.1 % or less. Decrease in assay value by 5.0% in accelerated stability testing is considered to be significant. Hence, it is very necessary to have analytical technique, which can detect decrease in assay and increase in impurities. Also the method should be efficient and reproducible.

Looking at various stringent quality requirements, high performance liquid chromatography (HPLC) has been considered as the most versatile technique. This is because of the availability of different types of stationary phases, unlimited choice of mobile phases, variety of detectors to be chosen from a range, very high throughput automation available and as a whole, applicability to the wide range of

compounds, which can not be analysed using other classical analytical techniques or even other form of chromatography because of their non specificity, less sensitivity and less efficiency. There are a number of books[59-67] and reviews[68-73] available on this subject discussing various aspects of the technique in detail. Many international symposia and conferences are held on this subject, which highlight the importance of this technique. It is therefore thought worthwhile to develop new analytical methods for analysing pharmaceutically important compounds and their formulations using the technique of HPLC.

1.6 DRUGS SELECTED FOR THE PROPOSED RESEARCH

For the proposed research work, following formulation and bulk drugs were selected.

1. Formulations drugs(Assays)

a. Candesartan

b. Captopril

c. Propanolol

d. Terazosin

e. Verapamil

2. Bulk drugs (Related substances/Impurity Profile)

a. Citalopram

b. Metaxalone

c. Ondansetron

CHAPTER - 2

THEORETICAL ASPECTS OF HPLC

2.1 CHROMATOGRAPHY

Chromatography[74-77] is essentially a physical method of separation in which the components to be separated are distributed between a stationary phase and a moving phase. The chromatographic process is the result of repeated sorption-desorption during the movement of the sample components along the stationary bed, and the separation is due to differences in distribution coefficients of individual sample components. Stationary phase is considered to be divided into many small zones called as theoretical plate and some amount of separation is supposed to be taking place at each theoretical plate before the component migrate. The distribution equilibrium is described by the distribution coefficient.

$$K_m = \frac{[X]_s}{[X]_m}$$

Where,

$[X]_s$ and $[X]_m$ are the concentrations of the component X in the stationary phase and mobile phase at equilibrium. Solutes with high Km values will be retained more strongly by the stationary phase than those with small Km values.

Therefore the latter will move along with column (be eluted) more rapidly.

2.2 CLASSIFICATION OF CHROMATOGRAPHIC TECHNIQUES

Chromatographic technique[78-79] is broadly classified into two categories as column chromatography and planar chromatography.

On the basis of type of stationary phase and the nature of mobile phase used, they can be further classified as shown in Table 2.1

Table 2.1 : Classification of chromatographic methods

Mobile Phase	Stationary Phase	Separation Mechanism	Technique	Chromatography
Gas	Liquid	Partition	Column	Gas liquid chromatography
Gas	Solid	Adsorption	Column	Gas solid chromatography
Gas	Solid	Molecular sieving	Column	Gas solid chromatography
Liquid	Liquid	Partition	Column	Classical liquid liquid chromatography
Liquid	Liquid	Partition	Planar	Thin layer chromatography
Liquid	Bonded	Modified liquid partition	Column	High performance liquid chromatography
Liquid	Bonded	Modified liquid partition	Planar	High performance thin layer chromatography
Liquid	Solid	Adsorption	Column	Liquid solid chromatography
Liquid	Solid	Adsorption	Planar	Thin layer and paper chromatography
Liquid	Solid	Ion exchange	Column	Ion exchange chromatography
Liquid	Solid	Exclusion	Column	Exclusion chromatography

2.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

In liquid chromatography[80-81] mobile phase is liquid and stationary phase is a solid or a liquid coated or bonded to a solid. Depending upon the kind of stationary phase used, the technique is referred as a liquid solid or liquid liquid chromatography. In contrast to gas chromatography, in liquid chromatography, very few stationary phases are available, whereas there is no limit to the choice of mobile phase and many solvents including water can be used depending upon the kind of stationary phase used. Also various buffers can be used to control pH of the mobile phase. A combination of 7 to 8 commonly used stationary phases and almost any combination of different organic solvents, use of buffers with different pH and ionic strength makes this technique a very fascinating to be exploited with almost any type of compounds. Another distinct advantage of this technique over gas chromatography is that it is normally carried out at room temperature, making it possible to be applied to thermally labile compounds. This technique is applicable to almost all class of pharmaceutical compounds.

2.4 SEPARATION MECHANISMS IN HPLC

Various separation mechanisms in HPLC[82] can be classified as follows:

1. Reversed phase chromatography
2. Normal phase chromatography
3. Ion pair chromatography.
4. Ion exchange chromatography
5. Size exclusion chromatography
6. Chiral chromatography

2.4.1 Reversed Phase Chromatography

It is the technique in which the stationary phase used is non polar and mobile phase is polar. This technique is most commonly used in pharmaceutical industries. Almost 90 % of all separations are carried out by reversed phase chromatography.

In reversed phase HPLC, separation is based on solvent strength. Column temperature, pH, flow rate, particle size of stationary phase and viscosity of mobile phase may also play an important role. In general, more polar components elute faster than less polar components. Compounds much more polar than the compound of interest may be masked (elute together) in the solvent front/void volume. Compounds very less polar than the analyte may elute either late during the chromatographic run or are retained in the column.

In this, the stationary phase is bonded chemically to the support surface.The stationary phases are prepared from rigid silica or silica based compositions. Now a days, some polymer based stationary phases are also available and are more stable over a wide range of pH. The most commonly used stationary phases in this type of chromatography are C-18 (octadecylsilane), C-8 (octylsilane). Other bonded phases used are C-2, C-4, cyano, amino and phenyl. Compounds with lower UV extinction coefficients or different wavelength maxima may not be detectable at the level relative to the visibility of the analyte since only one wavelength is normally monitored. But this can be avoided by using photodiode array detector in which the eluents can be monitored over the entire wavelength range simultaneously.

2.4.2 Normal Phase Chromatography

Normal phase chromatography is a chromatographic technique that uses organic solvents as mobile phase and a polar stationary phase. Here, the less polar components elute faster than the more polar components. Silica columns are generally used in this type of chromatography. Other stationary phases such as diol, cyano, amino and phenyl may also be used.

2.4.3 Ion exchange chromatography

Ion exchange chromatography refers to modern and efficient methods of separating and determining ions based upon ion-exchange[83] resins. Ion- exchange columns consist of particles of insoluble material containing positively or negatively charged functional groups. Cross-linked polystyrene is frequently used as the matrix with sulphonic acid groups attached for cationic exchanger and quaternary ammonium groups for anionic exchanger. If a mobile phase containing sample ions is passed through ion-exchange column, competitive distribution of ions between the resin and the mobile phase occurs. The rate of movement of these ions through the column varies depending on their relative affinities for the resin and is controlled by variety of factors such as charge on the ion, molecular weight of ionic species and pH of the mobile phase.

2.4.4 Ion pair chromatography

Ion pair chromatography[84] is the type of reversed phase partition chromatography that is used for the separation and determination of ionic species. Separation in ion pair chromatography is based on chemical interaction specific to the target species. The mobile phase consists of an aqueous buffer, organic solvent such as methanol

or acetonitrile and an added counter-ion of opposite charge to the sample capable of forming an ion-pair with the analyte ion. The interaction ion-pair thus formed is a neutral species, which can be retained by reversed phase packing. Factors such as ionic strength of the counter ion, pH of the mobile phase, temperature, concentration of and type of organic co-solvent (s) and dissociation constant of the analyte influence the separation. These separations are also characterized by sharper peak and reduced tailing for the analyte as compared to that obtained without the use of ion pairing agent. This is very useful in separation of mixture of compounds with different functionality such as acidic or basic compounds and wide variation in molecular weight. The common ion pairing agents used in chromatography of basic compounds are sodium salt of pentane, hexane, heptane and octane sulphonic acids whereas that for acidic compound is tetrabutyl ammonium hydroxide in methanol. Typical concentration that can be employed to effect the separation is 2.5 -10 mM. It takes more time to equilibrate the column for this type of separation. After carrying out separation, column should be washed properly; otherwise there is possibility of precipitation of residual counter ion at the time of next analysis, thus damaging the column. Sometimes there is a possibility of change in the retention behavior of the column due to improper washing.

2.4.5 Size Exclusion Chromatography[85]

This is also known as gel permeation or filtration chromatography. This is particularly applicable to high molecular weight species. Packing for size exclusion chromatography consists of small silica or polymer particles containing a network of uniform pores, into which solute and solvent molecules can diffuse. Separation is

based on the molecular size or hydrodynamic volume of the components. Molecules that are too large for the pores of the porous packing material on the column elute first, small molecules that enter the pores elute last, and the elution rates of the rest depend on their relative sizes.

2.4.6 Chiral Chromatography[86-88]

Separation of enantiomers can be achieved on chiral stationary phases by formation of diastereomers via derivatizing agents or mobile phase additives on achiral stationary phases. When used as an impurity test method, the sensitivity is enhanced if the enantiomeric impurity elutes before the enantiomeric drug[84].

2.5 THEORY OF HPLC

To understand the theory of chromatography[78,89], it is essential to consider the column as made up of number of small discrete and continuous units, called theoretical plate. At each plate, equilibrium of solute species between the stationary and the mobile phase is assumed to take place. A representative separation achieved in HPLC is shown in Figure 2.1.

For high separation efficiency, a large number of theoretical plates are necessary per unit length of the column. Number of theoretical plates is given by the equation:

$n = 16 (t / W)2$

where,

n=Number of theoretical plates

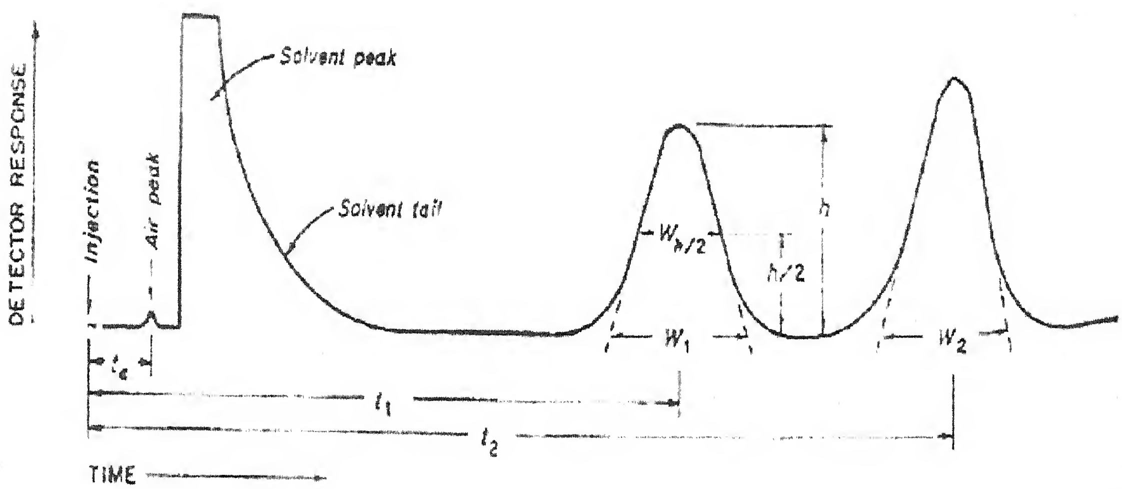
t = Retention time of component

w = Width of the base of the peak obtained by extrapolating the relatively straight

sides of the peak to the base line, where t and w both are reported in the same unit

As seen from the above equation, the number of theoretical plates are proportional to the retention time of the compound under consideration and inversely proportional to the width of the peak.

Figure 2.1 : **Representative separation in HPLC**



Another way of expressing the. efficiency of column is to calculate the height equivalent to a theoretical plate (HETP) which is the length of the column in cms divided by number of theoretical plates. HETP can be defined as that length of the column where separation between the constituent of a sample matrix takes place before they pass on to the next plate. Lower the HETP, higher is the efficiency of the column. HETP is calculated as:

$H = L/n$

where,

n=Number of theoretical plates

L = Length of the column

H = Height equivalent to a theoretical plate

Efficiency of the column increases as the number of theoretical plates are increased (i.e. H is decreased).

2.5.1 Capacity Factor

The capacity factor K' is defined as:

$$K' = \frac{t_1 - t_0}{t_0}$$

where,

t_1 = Retention time of the component

t_0 = Retention time of non retained component (i.e. void volume)

2.5.2 Resolution

The resolution R is a quantitative measure of the separation of two analyte peaks and is defined as:

$$R = \frac{2\,(t_2 - t_1)}{w_1 + w_2}$$

Where,

t_1 and t_2 are the retention times of the two components

w_1 and w_2 are the corresponding widths of the bases of the peaks, obtained by extrapolating the relatively straight sides of the peaks to the base line

2.5.3 Selectivity or Separation Factor (α)

Selectivity α, describes how well a chromatographic system can separate two compounds and it is defined as:

$$\alpha = \frac{t_2 - t_0}{t_1 - t_0}$$

Thus the resolution between the two peaks can be increased by increasing K', α or n, depending upon the purpose of separation (quantification, isolation) and the case with which the change is effected.

2.5.4 Van-Deempter Equation

Van-Deempter equation was originally established for gas chromatographic technique. But it has been shown that, with little variation it is also valid for liquid chromatography. It is expressed in terms of height equivalent to a theoretical plate.

$$HETP = A + B/\mu + C\mu$$

Where A, B and C are constants and μ, the linear flow rate (gas velocity in GC) through the chromatographic column. For a well-packed liquid chromatographic column, A, B and C are roughly constant[52].It suggests that the broadening of a peak is the summation of somewhat interdependent effects from several sources.

Constant A represents the eddy diffusion, which is due to the variety of tortuous (variable length) pathways available between the particles in the column and is independent of the gas or the mobile phase velocity. The constant A is characteristic of column packing and can be decreased with smaller and more uniform particles and tighter packing, which reduces the effective HETP and thus increases the efficiency.

Constant B represents the longitudinal or molecular diffusion of sample components in the mobile phase, due to concentration gradients within the column. The constant B is a function of both the sample and mobile phase. Since the sample components are fixed in a given analysis, the only way to change B is by varying the type, pressure and flow rate of the mobile phase. It can also be changed by decreasing the internal diameter of the column. In liquid chromatography, molecular diffusion in the stationary phase is very small as compared to that in gases.

Term C represents rate of mass transfer and is due to the finite time required for the solute equilibrium to be established between the two phases. The constant C is influenced by the partition coefficient, and therefore by the relative solubility of the sample in the stationary liquid phase (i.e. by the type and amount of liquid phase as well as the temperature). In case of adsorption chromatography, it is influenced by the adsorbing ability of the solute on the solid phase. Term C is decreased by decreasing the flow rate, allowing more time for equilibrium and also by keeping the stationary liquid phase film as thin as possible to minimize diffusion within this phase.

The reduced particle size of packing, uniform loading, thin film of stationary phase, controlled flow rate and low viscosity of mobile phase are the parameters which minimizes H, thereby increasing the column efficiency. The reduced plate height (H) helps in quick and rapid equilibrium of solute between stationary phase and mobile phase. The band broadening is reduced giving rise to sharp peaks.

2.6 INSTRUMENTATION USED IN HPLC

The basic components of HPLC instrument can be divided into 5 main

parts as:

. Solvent Management System

. Sample Management System

. Column

. Detector

. Data Collection

2.6.1 Solvent Management System

In order to provide very accurate and reproducible flow rates through the columns packed with particles of 3 to 10 µm sizes, which are very common in high performance liquid chromatography, pumping pressure of upto several thousand pounds per square inch is required. Following are the basic requirements for an HPLC pumping system:

. Capability to generate pressure upto 6000 psi

. Made up of corrosion resistant and inert material

. Pulse free flow rate ranging from 0.1 ml to 10 ml / min.

. Flow reproducibility of 0.5 %, or better

Two different types of pumps used in modern day HPLC equipment are

reciprocating pumps and displacement pumps.

a) Reciprocating pumps

These are used in most of the HPLC systems (about 90%). It consists of 2 small chambers called pump head, in, which the solvent is pumped by the back and forth motion of motor driven piston. When one of the piston is moving out of the pump

head to draw fresh mobile phase, the other is moving into the other chamber to drive out previously drawn mobile phase. The movement of these two pistons is such synchronized that the mobile phase flow to the injector is never stopped. But because of the inherent design, some amount of pulsation in the range of 10 to 15 psi is always observed. These pumps can produce pressure upto 6000 psi. They are readily adaptable to gradient elution and can produce constant flow rates.

b) Displacement pumps

Displacement pumps usually consist of large, syringe like chamber equipped with a plunger which is activated by a screw driven mechanism powered by a stepper motor. These pumps are used mainly with LC-MS systems, which require pulse less flow.

It is also very important to take into consideration the quality of solvents and mobile phases to be used in HPLC. Some of the most important criteria for the mobile phases are:

. Transparent in the UV region at least down to 210 nm or very low absorptivity

. Free from any particulate matter

. Free from dissolved gases

. Low viscosity

2.6.2 Sample Management System or Injector

The function of injector is to introduce the sample onto the top of the column in a narrow plug to prevent band broadening under high pressure. An improper injector becomes a limiting factor in the precision of results obtained.

Two types of injectors are used.

a) Syringe / Septum injector

These are used very rarely now a day. With these injectors, direct injection on a column with a micro syringe can be achieved.

b) Valve injector

These are most commonly used in HPLC systems. It injects fixed volume of sample solution. It has interchangeable loops providing a choice of sample sizes from 5 µl to 500 µl. It can introduce samples at pressure upto 6000 psi.

2.6.3 Column

Column is the heart of any chromatographic separation. Typical columns are made from highly polished stainless steel tubing which has a very uniform internal diameter. Typical column lengths are 10 to 30 cms, with an internal diameter of 2.0-4.6 mm. Most of the column packing in HPLC uses silica as the base material. Silica is either used as stationary phase or as a base material to bond other stationary phases[90] to it because of its higher mechanical strength.

Two different types of particles namely, total porous microspheres and micropellicular particles, are employed to prepare these stationary phases:

Porous microspheres are most commonly used particles because of the favorable compromise of desired properties such as efficiency, sample loading, durability and availability.

Micropellicular particles have a solid core with a very thin outer skin of interactive stationary phase. They are usually available in the range of 1.5 to 2.5 µm sizes. They display very high efficiency because of very fast mass transfers[91].

Separations using HPLC were carried out in early days on silica columns, but

because of various drawbacks such as its solubility at higher pH and surface acidity associated with some silica, these columns are replaced with more stable and versatile phases.

Different types of bonded phases using silica as the base material have being used instead of silica itself. Widely used column packing with different functionality is summarised in Table 2.2.

Table 2.2 : Common stationary phases used in HPLC

Sr No.	Stationary Phase	Application
1.	Silica	Adsorptive, used in normal phase
2.	Octadecylsilane (C-18)	Reversed phase, highly retentive, widely available
3.	Octylsilane (C-8)	Similar to C-18 but slightly less retentive
4.	Cyano (CN)	Moderately retentive, used for both reverse and normal phase chromatography
5.	Amino (NH2)	Highly polar, less stable, used for both reversed and normal phase chromatography
6.	Phenyl (C_6 H_5)	Moderately retentive, generally used in reversed phase chromatography
7.	Polystyrene	Used widely for size exclusion chromatography

2.6.4 Detector[92-93]

It is an eye of the whole HPLC system. Its function is to detect the compound eluted from the column and provide an electrical signal proportional to the concentration of the solute to the recorder. Some of the basic criteria required for detectors are outlined below:

1. High sensitivity

2. Higher linear dynamic range

3. Applicable to most of the solutes

4. Do not contribute to band broadening.

5. Non destructive

6. Faster response

The most common liquid chromatographic detectors are as follows:

a) UV-Visible detector (Absorbance detector)

These are the most versatile and commonly used detectors. The principle used in these detectors is same as that used for normal UV-Visible spectrophotometers. It allows the detection of various compounds from microgram to nanogram range depending on the molar absorptivity of compound.

Various types of UV-Vis detectors are available which are discussed below:

i) Fixed wavelength detector

These are the oldest detectors developed for HPLC. These detectors are tuned to a particular wavelength, generally 254 nm.

ii) Variable wavelength detector

These detectors provide a wavelength ranging from 200 to 800 nm employing deuterium or xenon lamp. The polychromatic light beam passes through the entrance slit and strikes the grating. Light of a particular wavelength coming from the grating is allowed to pass through the exit slit and flow cell and to strike the photomultiplier tube. The selection of wavelength is done by grating adjustment automatically.

iii) Photo diode array detectors[94] (PDA)

These are the latest absorbance detectors developed for HPLC over the last 10 years. In principle they are constructed similar to the variable wavelength detectors, but grating is fixed and situated after the sample. Instead of photomultiplier tube they have photodiode elements, which are placed very near to each other so as to form an array of tiny detectors. The distance between the two diodes and the optical design decides the overall resolution of the detector. These detectors have revolutionized the way in which the data is collected from HPLC separations. Using these detectors, it is possible to collect the spectral information of all components of a mixture as they are separated by chromatography. Detectors with wavelength resolution upto 1 nm are available in market. Using these detectors, it is possible to ascertain the quality of separation achieved by determining the purity of each peak. Also it is possible to monitor the response of different peaks at their individual wavelength maxima in order to get the optimum sensitivity for each of them. Earlier developed photo diode array detectors had little lower sensitivity levels than the variable wavelength detectors, but now with the advancement in technology, they have been brought at par. It is very much likely that in near future photo diode array detectors will become mandatory in order to have more reliability of data generated from HPLC analysis.

b) Fluorescence detector

In most of the fluorescence detectors, a photoelectric detector located at 90° angle to the excitation beam measures fluorescence. This sophisticated detector employs a xenon excitation source, and a grating monochromator to isolate a band of emitted

radiation. Detectors of this type are suitable for the detection of either fluorescent compound or non-fluorescent substances, which can be converted to fluorescent derivatives.

c) Refractive index detector

It is the universal detector useful for any non-UV absorbing compound. As the solute passes through the flow cell, the refractive index of solvent in the sample compartment changes, which is proportional to the concentration of the solute. The detector is sensitive to very small changes in mobile phase composition, temperature, pressure etc., and requires the use of degassed solvents delivered by pulse less flow delivery system.

However, the main disadvantage of refractive index detector is its low sensitivity. Contemporary refractive index detectors employing built in thermal control can reach sensitivity in the nanogram range.

d) Electrochemical detector

Electrochemical detectors can be used for the detection of electroactive compounds. It is based on the principle that electroactive species undergo electrolysis at an electrode when a suitable voltage is applied and that such analytes may be detected by monitoring the resulting current.

Other detectors though less widely used are mass spectrophotometer coupied to a liquid chromatograph (LC-MS) and light scattering detector. At present, the applications of LC-MS are limited to the identification of unknown impurities in a sample and to the biological sample in order to achieve a better sensitivity. But with the improvement in instrument design and cutting down the cost, it is likely to become a quality control tool like photo diode array detector in near future. Conductometric

detectors and chiral detectors have also appeared in market very recently for very special applications.

2.6.5 Data Collection

The signal produced during the detection of analyte is sent to an integrator, or more recently to software. This signal is measured and correlated to the quantity of the analyte present in the sample. Integrators of older days have been now a day replaced by very powerful software, which not only helps in processing the data precisely but also stores them for any evaluation at a latter stage, which is the basic regulatory requirement.

2.7 OPTIMIZATION OF HPLC METHODS

As discussed earlier in the preceding chapter, HPLC has been the most useful and versatile among all chromatographic techniques, because of its wide applicability to different classes of compounds and the sensitivity levels achieved.

There has been considerable improvement in the hardware used since its invention. Major improvement in this field is due to availability of columns with 3-5 μ particle sizes, instead of 30 μ columns used earlier[95]. HPLC has found widespread applications in pharmaceutical industry. Various types of pharmaceutical analyses such as drug purity determination, process monitoring of bulk drug, assay and dissolution testing of formulation etc., requires a very versatile, sensitive and reliable technique like HPLC. Ultra high resolution is the requirement for assay and stability studies, whereas faster analysis with moderate resolution is the requirement for dissolution testing. The requirement for bio studies is faster analysis with higher resolution and better sensitivity. All these requirements can be met by optimization of one or more of the following[96]:

1. Choice of column stationary phase

2. Separation temperature

3. Solvent strength and solvent type

4. Type of modifier

Various chromatographic scientists have studied the effect of different stationary phases[97-101] effect of solvent strength[102-107] and temperature[108]. Original developments carried out using normal phase chromatography on silica columns have been gradually replaced by reverse phase separations due to the versatility associated with the latter technique[109].

The most common stationary phase that has been used is the octadecylsilane chemically bonded to the silica using different bonding and end capping techniques. There has been a biased trend towards this stationary phase and almost 50 % of the total reverse phase separations carried out today are reported using this stationary phase[110]. Various stationary phases such as silica bonded with phenyl and cyano alkyl functionalities have been developed subsequent to the development of octadecylsilane columns. Although these stationary phases have a great potential in terms of the three most sought after parameters in chromatography namely selectivity- sensitivity, resolution and speed, they have been hardly exploited for this purpose. Some scientists have worked and reported various optimized conditions for separation of different mixtures. Optimization of different parameters helps in achieving these goals. In the present work, HPLC technique has been employed for method development of bulk drugs and formulations. The results have been presented and discussed separately in the respective chapters.

CHAPTER - 3

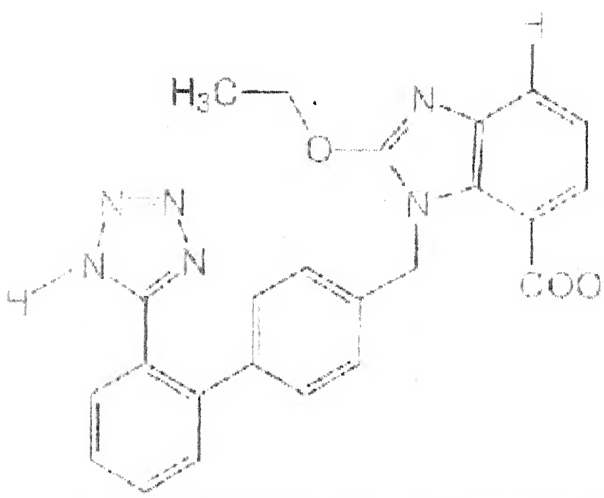
REVIEW OF LITERATURE AND RESEARCH ENVISAGED

Literature survey for each of the drugs of the selected formulations and bulk drugs revealed the following information on general properties and methods of analysis in bulk, in single and multi-component formulations and in bio-fluids.

3.1 CANDESARTAN [111-114]

3.1.1 General Information

Structure:



Chemical Name : 2-ethoxy-3-[[4-[2-(2H-tetrazol-5-yl)phenyl]phenyl]methyl]-3H-benzoimidazole-4-carboxylic acid.

Molecular Formula : $C_{24}H_{20}N_6O_3$

Molecular Weight : 440.45

Description : White to off white powder.

Solubility : Practically insoluble in water and sparingly soluble in methanol.

Category : Antihypertensive.

3.1.2 Reported methods of analysis

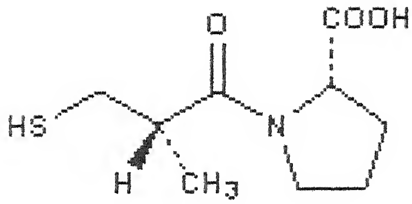
Sr.No	Method	Sailent Features	Matrix	Ref. No.
1.	HPLC-Fluorescence detection	Cyano column,buffer tetrabutyl ammonium hydrogen sulphate, acetonitril and methanol as mobile phase, fluorescence detection at 265 nm and 395 nm.	Human plasma/ urine	115
2.	Voltammetry	The electrochemical behaviour of candesartan cilexetil (CND) was investigated in an acetonitrile: supporting electrolyte mixture (30% acetonitrile) in the pH range 1.5-11.00 by cyclic, linear sweep, differential pulse (DPV), adsorptive stripping differential pulse (AdSDPV), square wave (SWV) and adsorptive stripping square wave (AdSSWV) voltammetric techniques. CND exhibited one wave and one peak to the anodic direction. The oxidation process was found to be irreversible and adsorption controlled. To obtain good sensitivity, the instrumental and accumulation variables were studied using DPV and SWV techniques. Two linear calibration plots were obtained for both techniques. The detection limits were 9.15 x 10(-7) M and 7.94 x 10(-6) M for AdSDPV and AdSSWV, respectively. The method was validated and successfully applied for the analysis of CND tablets.	Formul-ation	116
3.	HPLC	The system requires a Supelcocil C[18] (5 µm, 15 cm x 4.6 mm) column, and a mobile phase composed of 10 mM potassium dihydrogen phosphate: methanol: acetonitrile (2: 80: 18, v/v/v) (pH 2.5) while at a flow rate 1.0 mL min[-1].detector 260 nm, The method utilises protein precipitation	Formul ation/Pl asma	117

		with acetonitrile as the only sample preparation involved prior to reversed phase-HPLC. No internal standard was required.		
4.	U.V-Spectrophotometry	Two-component mixtures of candesartan cilexetil (CAN) and hydrochlorothiazide (HYD) were assayed by first derivative and ratio derivative spectrophotometry. The first method depends on zero-crossing and peak to base measurement. The first derivative amplitudes at 270.1 and 255.5 nm were selected for the assay of (CAN) and (HYD), respectively. The second method depends on first derivative of the ratio spectra by division of the absorption spectrum of the binary mixture by a normalized spectrum of one of the components and then calculating the first derivative of the ratio spectrum. The first derivative of the ratio amplitudes at 236, 250, 232, 267 and 280 nm were selected for the determination of (CAN) and (HYD), respectively.	Formul at-ion	118
5.	HPLC-Fluorescence detection	The chromatography was carried on a reversed-phase column, μ Bondapak C[18], at room temperature. A gradient elution mode was used to carry out the separation, the optimal mobile phase being composed of acetonitrile-5 mM acetate buffer, pH 4, at variable flow-rates (from 1.0 to 1.2 ml/min). Fluorescence detector was set at an excitation wavelength of 250 nm and an emission wavelength of 375 nm. internal standard (bumetanide,) was used	Human plasma	119

3.2 CAPTOPRIL [120-122]

3.2.1 General Information

Structure:



Chemical Name : (2S)-1-[(2S)-2-methyl-3-sulfanyl-propanoyl] pyrrolidine-2-carboxylic acid

Molecular Formula : $C_9H_{15}NO_3S$

Molecular Weight : 217.29

Description : Almost white to cream coloured crystalline powder.

Solubility : Freely soluble in water, alcohol, chloroform and methylene chloride

Category : Antihypertensive.

3.2.2 Reported methods of analysis

Sr.No	Method	Sailent Features	Matrix	Ref. No.
1.	HPLC-Derivatisation	Captopril was derivatized with 2,4′-dibromoacetophenone (pBPB) to form a captopril-pBPB adduct. From acidified serum plasma samples, the hydrochlorothiazide and derivatized captopril was extracted with 5 ml ether, then with 5 ml dichloromethane. Effective chromatographic separation was achieved using a C18 column (DIAMONSIL 150 mm × 4 mm i.d.,	Human plasma	123

		5 µm) based on an acetonitrile–trifluoroacetic acid–water gradient elution at a flow rate of 1.2 ml/min. The internal standard (IS), derivatized captopril and hydrochlorothiazide were detected at 263 nm and were eluted at 4.2, 6.8 and 16.9 min, respectively.		
2.	HPLC-Indirect photometric detection	A low capacity anion-exchange column was used with potassium phthalate as the mobile phase marker and indirect detection at 280 nm. The chromatographic conditions were optimized using the Box and Behnken factorial experimental design	Formulation	124
3.	HPLC	The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with stationary phase C (10 µm) (Nucleosil C18 is suitable), (b) a mixture of 0.5 volume of orthophosphoric acid, 450 volumes of water and 550 volumes of methanol as the mobile phase with a flow rate of 1 ml per minute and (c) a detection wavelength of 220 nm.	Formulation	125
4.	Spectroscopy	Dissolve about 300 mg of Captopril, accurately weighed, in 100 mL of water in a suitable glass-stoppered flask, add 10 mL of 3.6 N sulfuric acid, 1 g of potassium iodide, and 2 mL of starch TS. Titrate with 0.1 N Potassium iodate titrant to a faint blue endpoint that persists for not less than 30 seconds. Perform a blank determination (see Titrimetry á 541 ñ), and make any necessary correction. Each mL of 0.1 N Potassium iodate titrant is equivalent to 21.73 mg of C 9H 15NO 3S.	Formulation	126
5.	Volumetric and spectrophotometric	The methods were based on the reaction of captopril with potassium iodate in HCl medium.	Formulation	127

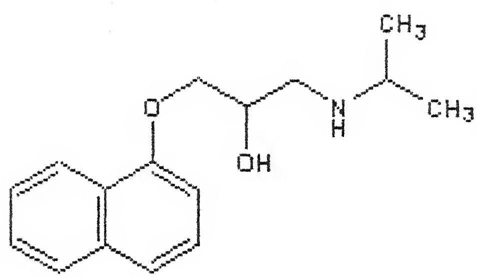
		Amaranth was used as indicator to detect the end-point of the titration in aqueous layer. The iodine formed during the titration was extracted into CCl4 and subsequently determined spectrophotometrically at 510 nm. The Beer's law was obeyed in the concentration range of 120–520 µg ml−1.		
6.	CE method	This study describes the development of a CE method for the analysis of the antihypertensive drug captopril using LIF detection. The method is based on the derivatization of captopril with the fluorescent label 5-iodoacetamidofluorescein. The optimization of the electrophoretic electrolyte composition together with other variables, such as applied voltage and injection time, resulted in a solution of 20 mM phosphate buffer adjusted to pH 12.0	Formulations/ Urine	128
7.	FT-Raman spectroscopy	A procedure for the quantitative determination of captopril and prednisolone in commercial tablets based on partial least squares (PLS) and principal component regression (PCR) treatment of FT-Raman spectroscopic data Results obtained from calibration models built using unnormalised spectra were compared with the values found when an internal standard was added to each sample and the spectra were normalised by its selected band intensity at maximum or integrated.	API/Formulation	129
8.	Colorimetric and potentiometric	The development of an analytical procedure based on the sequential injection analysis (SIA) concept for the analytical control of captopril in pharmaceutical formulations is described. One of the captopril determination procedures is based	Formulation	130

		on the colorimetric measurement of the captopril–palladium complex in acid media at the 400 nm. the potentiometric titration of captopril with a 10−3 mol/l Ag(I) solution used as titrant and the application of the Gran's method for ascertaining the equivalence point.		
9.	indirect biamperometric detection	A flow injection manifold based on the indirect biamperometric detection of the captopril by using Fe(III)/Fe(II) as an indicating redox system and a Z-shaped flow-cell configuration, was developed.	Formulation	131

3.3 Propranolol [132-134]

3.3.1 General Information

Structure:



Chemical Name : 1-(1-methylethylamino)-3-naphthalen-1-yloxy-propan-2-ol

Molecular Formula : $C_{16}H_{21}NO_2$

Molecular Weight : 259.343 g/mol

Description : White to off white crystalline powder.

Solubility : Soluble in water, alcohol. Practically insoluble in ether, Benzene, ethyl acetate.

Category : Antihypertensive.

3.3.2 Reported methods of analysis

Sr.No	Method	Sailent Features	Matrix	Ref. No.
1.	solid phase extraction and capillary zone electrophoresis	Determination of propranolol (a beta-blocker) and one of its metabolites, N-desisopropyl propranolol, has been developed and validated. The optimum separation of both analytes was obtained in a 37 cm × 75 µm fused silica capillary using 20 mmol/L phosphate buffer (pH 2.2) as electrolyte, at 25 kV and 30 °C, and hydrodynamic injection for 5 s. Prior to the electrophoretic separation, the samples were cleaned up and concentrated using a C18 cartridge and then, eluted with methanol, allowing a concentration factor of 30.	Human urine	135
2.	HPLC-Fluorescence detector	Determination of propranolol concentration in the small volume of rat plasma without the solvent extraction step using pronethanol as the internal standard. The analysis was accomplished using a 5 µm CAPCELL PAK analytical cyano column at room temperature and a mobile phase consisted of 1% aqueous acetic acid containing 0.2% triethylamine and acetonitrile (65:35, v/v; pH 3.8). The flow-rate was kept at 0.5 mL/min and column effluent was monitored with a fluorescence detector at an excitation wavelength of 230 nm and an emission wavelength of 340 nm.	Rat plasma	136
3.	HPLC	Dissolve 0.5 g of sodium dodecyl sulfate in 18 mL of 0.15 M phosphoric acid, add 90 mL of acetonitrile and 90 mL of methanol, dilute with water to make 250 mL, mix, and equipped	Formulation	137

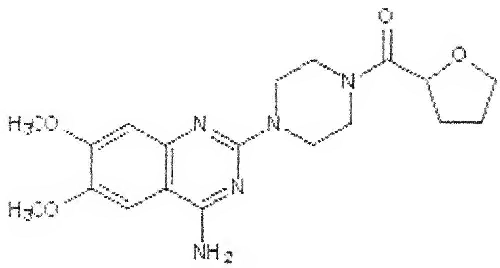
		with a 290-nm detector and a 4.6-mm × 25-cm column that contains 5-µm packing L7.The flow rate is about 1.5 mL per minute.		
4.	Spectroscopy	Shake a quantity of the powder containing 20 mg of Propranolol Hydrochloride with 20 ml of water for 10 minutes. Add 50 ml of methanol, shake for a further 10 minutes, add sufficient methanol to produce 100 ml and filter. Dilute 10 ml of the filtrate to 50 ml with methanol and measure the absorbance of the resulting solution at the maximum at 290 nm, Appendix II B. Calculate the content of C16H21NO2,HCl taking 206 as the value of A(1%, 1 cm) at the maximum at 290 nm.	Formulation	138
5.	HPLC	A HPLC procedure was established to assay propranolol enantiomers in rat hepatic microsomes. After the termination of the reaction with methanol. 10µL of the sample was applied to a reversed phase column (Shim-pack CLC-ODS 15cm×0.6cm id, 10µm parti cle size). Propranolol was monitored with a UV detector at 290nm. The mobile phase was made up with ammonium acetate buffer (pH 4.0)-methanol (50:50). The flow rate was 1.0mL/min.	Rat hepatic microsomes	139
6.	HPLC-Fluorescence detector	Propranolol was analyzed by a validated HPLC method using fluorescence detection and solid-phase extraction. The analytical column used was a Zorbax C-8 reverse-phase column (Mac-Mod Analytical, Inc., Chadds Ford, PA), and the mobile phase consisted of 0.25% phosphoric acid and acetonitrile (74:26, vol/vol).	Formulation and Human blood plasma	140
7.	HPLC	55% 67 mM potassium phosphate buffer with 0.2% triethylamine, pH adjusted to 7.0,flow rate 0.8	Formulation	141

		ml/min, detector set at 294 nm		
8.	HPLC-Fluorescence detector	An automated HPLC method is described for the simultaneous determination of propranolol, 4-hydroxypropranolol, and N-desisopropylpropranolol in plasma and urine before and after beta-glucuronidase/aryl sulfatase treatment. It involves extraction with ether at pH 10 in the presence of ascorbic acid, added to prevent oxidation of 4-hydroxypropranolol. The compounds are then back extracted into dilute acid and assayed on an HPLC using a fluorescence detector. Three HPLC columns have been used (a phenyl, an octyl, and an octadecyl column). The last column was found to be most reproducible with minimal intercolumn variation. The solvent system includes a combination of acetonitrile, methanol, and phosphoric acid. Concentrations as low as 0.2, 1.0, and 0.2 ng/ml of propranolol, 4-hydroxypropranolol, and N-des isopropyl propranolol, respectively, can be measured using 1 ml of plasma.	Plasma /urine	142
9.	Spectroscopy	Two simple and sensitive indirect spectrophotometric methods for the assay of propranolol hydrochloride (PPH) and piroxicam (PX) in pure and pharmaceutical formulations have been proposed. The methods are based on the oxidation of PPH by a known excess of standard N-bromosuccinimide (NBS) and PX by ceric ammonium sulfate (CAS) in an acidic medium followed by the reaction of excess oxidant with promethazine hydrochloride (PMH) and methdilazine hydrochloride (MDH) to yield red-colored products.	Formul ation	143

3.4 TERAZOSIN[144-146]

3.4.1 General Information

Structure:



Chemical Name : [4-(4-amino-6,7-dimethoxy-quinazolin-2-yl)piperazin-1-yl]-tetrahydrofuran-2-yl-methanone

Molecular Formula : $C_{19}H_{25}N_5O_4$

Molecular Weight : 387.433

Description : White to pale yellow crystalline powder.

Solubility : Freely soluble in water, methanol, slightly soluble in ethanol and practically insoluble in hexane.

Category : Antihypertensive.

3.4.2 Reported methods of analysis

Sr.No	Method	Sailent Features	Matrix	Ref No.
1.	HPLC	The liquid chromatograph is equipped with a 254-nm detector and a 4.6-mm × 25-cm column that contains packing L7. The column temperature is maintained at about 30°. The flow rate is about 1.0 mL per minute. pH 3.2 Citrate buffer is prepared by dissolving 12.0 g of	Formulation	147

		sodium citrate dihydrate and 28.5 g of anhydrous citric acid in 1.95 L of water. Adjust with anhydrous citric acid or sodium citrate to a pH of 3.2 ± 0.1.Dilute with water to 2.0 L, and mix. Mobile phase is mixture of pH 3.2 Citrate buffer and acetonitrile (1685:315).		
2.	HPLC	The present paper describes development of stability-indicating high performance liquid chromatographic (HPLC) assay methods for three alpha-adrenergic-blocker drug substances, namely, prazosin, terazosin and doxazosin, in the presence of degradation products generated from forced decomposition studies. Resolution of drugs from degradation products was obtained using a reversed-phase C-18 column using water/acetonitrile/methanol/glacial Acetic acid /diethylamine (25:35:40:1:0.017) as mobile phase for prazosin and terazosin The detection was done at 254 nm. The methods were validated with respect to linearity, precision, accuracy, specificity and robustness.	Formulation	148
3.	Voltametry	The electrochemical behavior of terazosin at the hanging mercury drop electrode was studied in Britton–Robinson buffer (pH 2–11), acetate buffer (4.5–5.5), and in 0.1 M solution of each of sodium sulfate, sodium nitrate, sodium perchlorate and potassium chloride as supporting electrolytes. The square-wave adsorptive cathodic stripping voltammogram of terazosin exhibited a single well-defined two-electron irreversible cathodic peak which may be attributed to the reduction of C=O double bond of the drug molecule. A fully validated, simple, high sensitive, precise and inexpensive square-wave adsorptive cathodic stripping voltammetric	Human Plasma Formul-ation Bulk drug	149

		procedure was described for determination of terazosin in bulk form, tablets and human serum. A mean recovery for 1×10−8 M terazosin in bulk form, following pre concentration onto the hanging mercury drop electrode for 60 s at a −1.0 V (versus Ag/AgCl/KCls), of 99±0.7% (n=5) was obtained. Limits of detection (LOD) and quantitation (LOQ) of 1.5×10−11 and 5×10−11 M bulk terazosin were achieved, respectively.		
4.	Frequency-domain photon migration (FDPM) measurements of time-dependent light propagation	Frequency-domain photon migration (FDPM) measurements of time-dependent light propagation are conducted to provide the powder absorbance for quantitative prediction of terazosin as the active pharmaceutical ingredient (API) in a low-dose (0.72 wt %) oral tablet formulation. Calibration of the FDPM-derived powder absorbance at discrete wavelengths of 514, 650, 687, and 785 nm was performed for API contents ranging between 0 and 1.5 wt % in mixtures showing maximum sensitivity at 650 nm. The relative standard deviation (RSD) of FDPM absorption coefficient measurement at 650 nm in a well-mixed 1.08 wt % terazosin blend was <1.6%, of which no more than 0.12% arose from FDPM instrumental error and the remainder was attributable to the complete-random-mixture model. The applicability of FDPM as an on-line sensor for powder-blending operations was further evaluated by analyzing grab samples taken directly from five locations of a 2-cu-ft Gallay blender at intervals of 5 min within the blending process. FDPM results indicate that homogeneity was largely achieved in the first 10 min, during which the RSD of API content across five sampling	Formulation Bulk Drug	150

		locations decreased from 27% to 8%, and the RSD decreased to 5% after 25 min of blending. Evolution of homogeneity within the blending process assessed through FDPM measurements was fit to the first-order model of particle blending further evidencing applicability for monitoring powder-blending processes.		
5.	HPLC/Fluorescen-ce detector	A high-performance liquid chromatographic (HPLC) analysis of terazosin in 1 ml of human plasma was developed using prazosin as an internal standard. The plasma sample was extracted with dichloromethane and ethylether and a 100-micro aliquot was injected onto the reversed-phase column. The mobile phase, 0.02 M sodium phosphate buffer :acetonitrile : tetrahydrofuran 720:220:60 (v/v/v), was run at a flow rate of 0.8 ml/min and the column effluent was monitored using a fluorescence detector set at 370 and 250 nm for the emission and excitation wave numbers, respectively. The retention times for terazosin and prazosin were approximately 6.4 and 9.8 min, respectively.	Human Plasma	151
6.	Derivative spectro-photometry Colorimetry Fluorimetry	Five simple and accurate methods are presented for the determination of terazosin (TZ) in tablets. These methods are based on: the direct measurements of the first and second derivative spectra of samples (A), the reaction of TZ with chloranil (CH) in aqueous solution of pH 9 to give an intense yellow color measured at 340 nm (B), the reaction of the drug with mercurochrome (MER) in aqueous alkaline medium to give an intense red color measured at 543 nm (C), the formation of an ion-pair salt between the drug and bromocresol purple (BCP) with subsequent absorbance measurements at 412	Formul ation	152

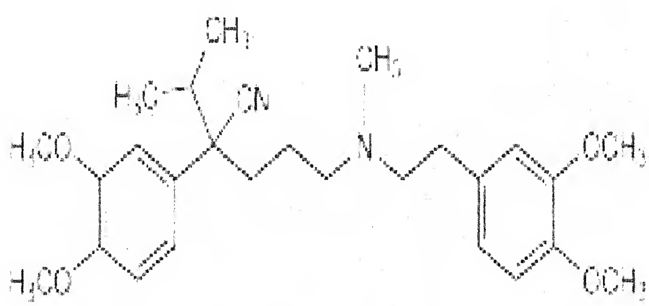
		nm (D), and a sensitive fluorimetric method (E). The latter method was extended to determine TZ in presence of its degradation products.		
7.	HPLC/Fluorescence detector	For terazosin content the samples were analysed by h.p.l.c. with fluorescence detection. The internal standard solution, aqueous dimethothiazine (5 mg l–1, 20 μl), NaOH solution (4 m, 50 μl) and saturated NaCl solution (100 μl) were added to plasma (50 μl). Terazosin and the internal standard were extracted into methyl tertiary-butyl ether (200 μl) by mixing for 30 s and centrifugation at 11 000 rev min–1 for 4 min. A portion (40 μl) of the extract was injected onto the h.p.l.c. column (mobile phase: 40 mm ammonium perchlorate in methanol: deionised water (9+1) adjusted to pH 6.8 with 1% (v/v) methanolic perchloric acid). Detection was by fluorescence monitoring with excitation at 250 nm. The limit of accurate measurement of the assay was 5 ng ml–1.	Plasma	153
8.	HPLC/Fluorescence detector	The chromatographic system consisted YMC basic 150 × 4.6 mm i.d. column (YMC, Wilmington, NC). The column was eluted with 20:80 v/v acetonitrile and 0.1% TFA at a constant flow rate of 0.8 ml/min at room temperature. The effluent from the column was monitored for fluorescence emission using a 350-nm filter after excitation at 250 nm. The retention times were 6.0 and 10.0 min for terazosin and prazosin, respectively. The standard curve was linear from 2 to 200 ng/ml (triplicate samples) with correlation coefficients ≥ .999. Coefficients of variation were determined for triplicate spiked samples at 2, 20 and 200 ng/ml and resulted in values of <10%. Interday coefficients of variation were also determined for the	Human Plasma	154

		spiked samples from three separate experiments and resulted in values of < 7%. On the basis of a coefficient of variation of < 20%, the assay had a quantitation limit of 0.5 ng/ml. Analysis of control blank plasma indicated the absence of interfering peaks.		
9.	HPLC	A rapid, selective and reproducible high performance liquid chromatographic method has been developed for the determination of terazosin in human plasma. Terazosin plus the internal standard, prazosin hydrochloride, were extracted from alkalified plasma with tert-butylmethyl ether, back-extracted into 0.05% phosphoric acid. Fifty portions of extract were injected onto a octadecylsilane column and eluted with a mixture of acetonitrile, water and triethylamine (30 : 70 : 0.1 v/v, adjusted to pH 5.0 with dilute phosphoric acid) at a flow rate of 1.0 ml/min. The fluorescence intensity of column eluents was monitored at excitation wavelength of 250 nm and emission wavelength of 370 nm. No interference peaks were observed. The practical limit of quantitation was 5 ng/ml for terazosin.	Human Plasma	155

3.5 Verapamil [156-159]

3.5.1 General Information

Structure:



Chemical Name : 2-(3,4-dimethoxyphenyl)-5-[2-(3,4-dimethoxyphenyl)ethyl-methyl-amino]-2-(1-methylethyl) pentanenitrile

Molecular Formula : $C_{27}H_{38}N_2O_4$

Molecular Weight : 454.602

Description : White crystalline powder.

Solubility : Soluble in water; sparingly soluble in alcohol; freely soluble in chloroform; practically insoluble in ether.

Category : Antihypertensive.

3.4.2 Reported methods of analysis

Sr.No	Method	Sailent Features	Matrix	Ref No.
1.	HPLC	A simple, specific, and accurate HPLC assay method is presented for the simultaneous measurement of the calcium antagonists nifedipine and verapamil in rat plasma. The plasma sample was deproteinized by treatment with an equivalent volume of an internal standard solution (nicardipine hydrochloride in acetonitrile containing 0.1% of perchloric acid), followed by brief centrifugation. A 50 µL aliquot of the clear supernatant was analyzed on a Microsorb-MV C18, 5 µm, column using a mixture of acetonitrile-methanol-0.01 M phosphate buffer pH 5.2 (55:15:30) as the mobile phase. At a flow rate of 1 mL/min and detection at 235 nm, nifedipine, verapamil, and nicardipine were observed to elute at about 3.4 min, 6.4 min, and 9.8 min, respectively.	Rat Plasma	160
2.	HPLC	A high-performance liquid chromatographic (HPLC) method	Rat Plasma	161

		was developed for the assay of verapamil in rat plasma. After deproteinization of the plasma sample with an acetonitrile-perchloric acid (8:2) mixture containing dextromethorphan, the internal standard, an aliquot of the supernatant was directly analyzed on a cyanopropylsilane column with methanol-acetonitrile-triethylamine acetate buffer (10:30:60) as the mobile phase and detection at 235 mm. At a flow rate of 1.5 ml min^-^1, a complete analysis was completed in less than 6 min.		
3.	HPTLC	A high-performance thin-layer chromatography (TLC) method coupled with densitometric analysis has been developed for simultaneous measurement of trandolapril (TRA) and verapamil (VER) in 2-component mixtures and in their combination capsules. The active substances were extracted from capsules with methanol (mean recovery: 103.4% for TRA, 97.13% for VER) and chromatographed on TLC plates coated with silica gel 60 F254 in horizontal chambers with ethyl acetatc-ethanol-acetic acid (8 + 2 + 0.5, v/v) mobile phase. Chromatographic separation of these components was followed by ultraviolet densitometric quantification at 215 nm.	Formulation	162
4.	Spectro-photometric	A rapid, simple and sensitive validated visible spectrophotometric method has been described for the assay of verapamil hydrochloride either in pure form or in pharmaceutical formulations. The method involves the oxidation of the verapamil hydrochloride with N-bromosuccinimide in perchloric acid medium at room temperature, leading to the formation of a yellow colored product, which absorbs maximally at 415 nm. Under the	Bulk/ Formulation	163

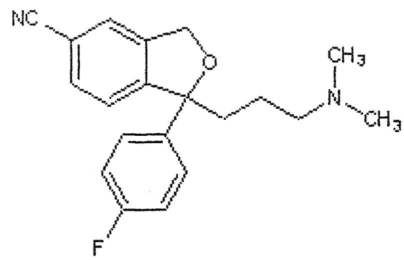
		optimized experimental conditions, the color is stable up to 45 min and Beer's law is obeyed in the concentration range of 10.0-200.0 microg ml(-1) .		
5.	LCMS	An analytical method based on liquid chromatography with positive ion electrospray ionization (ESI) coupled to tandem mass spectrometry detection (LC–MS/MS) was developed for the determination of Verapamil in human plasma using Metoprolol as the internal standard. The analyte and internal standard were extracted from the plasma samples by liquid–liquid extraction and chromatographed on a C8 analytical column. The mobile phase consisted of methanol–water (70:30; v/v) + 12 mM formic acid. The method had a chromatographic total run time of 3.5 min and was linear within the range 1.00–500 ng/mL. Detection was carried out on a Micromass Quattro Ultima tandem mass spectrometer by multiple reaction monitoring (MRM).	Human Plasma	164
6.	HPLC-Fluorescence detector	Verapamil, was determined in serum and urine samples by a sensitive and precise chromatographic procedure without any pre-treatment step in a C18 column using a micellar mobile phase of 0.15 M sodium dodecyl sulfate and 5% pentanol at pH 7. Fluorescence detection set at 230 nm (excitation) and 312 nm (emission) was used. Verapamil is eluted at 12.5 min with no interference by the protein band or endogenous compounds.The procedure developed can be useful in the field of toxicology and clinical analysis.	Serum and Urine	165
7.	HPLC	A simple, sensitive and specific reversed phase high performance liquid chromatographic (RP-HPLC) method with UV detection at 251 nm	Rat Plasma	166

		was developed for simultaneous quantitation of buparvaquone (BPQ), atenolol, propranolol, quinidine and verapamil. The method was validated on a C-4 column with mobile phase comprising ammonium acetate buffer (0.02 M, pH 3.5) and acetonitrile in the ratio of 30:70 (v/v) at a flow rate of 1.0 ml/min. The retention times for atenolol, quinidine, propranolol, verapamil and BPQ were 4.30, 5.96, 6.55, 7.98 and 8.54 min, respectively.. The method is simple, reliable and can be routinely used for accurate permeability characterization.		
8.	Voltammetry	A sensitive reduction peak of verapamil is obtained by adsorptive stripping voltammetry in 0.01 M phosphate (pH 7.4) at an accumulation time of 30 s. The peak potential is −1.81 V (vs. Ag/AgCl). The peak current is directly proportional to the concentration of verapamil (1×10−8–1×10−6 M), with a 3σ detection limit of 5×10−10 M (0.246 ng/ml). The R.S.D. at the 1×10−7 M level is 1.8%. The method is simple (no extraction), rapid (30 s accumulation time), sensitive (the detection limit of verapamil is 0.491 ng/ml), reproducible(within day R.S.D. of 1.28–1.8%),	Urine/Formulation	167
9.	Spectrophoto-metry	Weigh and powder 20 tablets. Shake a quantity of the powder containing 0.1 g of Verapamil Hydrochloride with 150 ml of 0.1M hydrochloric acid for 10 minutes, add sufficient 0.1M hydrochloric acid to produce 200 ml and filter. Dilute 10 ml of the filtrate to 100 ml with water and measure the absorbance of the resulting solution at the maximum at 278 nm,. Calculate the content of C27H38N2O4,HCl taking 118 as the value of A(1%, 1 cm) at the maximum at 278 nm.	Formulation	168

3.6 CITALOPRAM[169-172]

3.6.1 General Information

Structure:



Chemical Name	: 1-(3-dimethylaminopropyl)-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5- carbonitrile
Molecular Formula	: $C_{20}H_{21}FN_2O$
Molecular Weight	: 324.392 g/mol
Description	: White to off white crystalline powder.
Solubility	: Sparingly soluble in water, soluble in ethanol, freely soluble in chloroform and very slightly soluble in diethyl ether.
Category	: Antidepressent.

3.6.2 Reported methods of analysis

Sr.No	Method	Sailent Features	Matrix	Ref. No.
1.	HPLC	A method for automated quantitative analysis of (es-)citalopram and desmethyl(es-)citalopram in serum using column-switching high performance liquid chromatography (HPLC). For sample clean-up serum was	serum	173

.		injected onto a LiChrospher CN 20 microm precolumn using 8% acetonitrile in deionized water. Drugs were eluted by back-flush flow onto the analytical column (LiChrospher CN 5 microm) at a flow rate of 1.5 ml/min with phosphate buffer 8 mmol/l pH 6.4/acetonitrile (50/50, v/v). Haloperidol was used as internal standard. Analytes were detected by ultraviolet spectrophotometry at 210 nm. Detection limit of (es-)citalopram was 6 ng/ml. The method was found to be suitable for therapeutic drug monitoring of patients treated with citalopram or escitalopram.		
2.	Capillary gas chromatography with mass spectrometry	Capillary gas chromatography with mass spectrometry detection in SIM mode (GC–MS-SIM) has been used for the analysis of citalopram (CIT), fluoxetine (FLX), and all of their metabolites in urine samples. The instrumental parameters affecting GC separation and MS-SIM detection were investigated. A validation procedure was performed on urine matrix and a simultaneous robustness evaluation is also presented in this paper.	Urine	174
3.	HPLC coupled to U.V and Fluorescence detector.	A method for the simultaneous determination of the three selective serotonin reuptake inhibitors (SSRIs) citalopram, fluoxetine, paroxetine and their metabolites in whole blood and plasma was developed. Sample clean-up and separation were achieved using a solid-phase extraction method with C8 non-endcapped columns followed by reversed-phase high-performance liquid chromatography with fluorescence and ultraviolet detection. The method was validated for the concentration range 0.050-5.0	Plasma	175

		micromol/l with fluorescence detection and 0.12-5.0 micromol/l with ultraviolet detection. The limits of quantitation were 0.025 micromol/l for citalopram and paroxetine, 0.050 micromol/l for desmethyl citalopram, di-desmethyl citalopram and citalopram-N-oxide, 0.12 micromol/l for the paroxetine metabolites by fluorescence detection, and 0.10 micromol/l for fluoxetine and norfluoxetine by ultraviolet detection. The method has been used for the analysis of whole blood and plasma samples from SSRI-exposed patients and forensic cases.		
4.	Capillary Electrophoresis	A newly developed disposable device for liquid-phase microextraction (LPME) was evaluated for the capillary electrophoresis (CE) of the antidepressant drug citalopram (CIT) and its main metabolite N-desmethylcitalopram (DCIT) in human plasma. CIT and DCIT were extracted from 1 ml plasma samples through hexyl ether immobilised in the pores of a porous polypropylene hollow fibre and into 25 microl of 20 mM phosphate buffer (pH 2.75) present inside the hollow fibre (acceptor phase). Prior to extraction, the samples were made strongly alkaline in order to promote LPME of the basic drugs. Owing to the high ratio between the volumes of sample and acceptor phase, and owing to high partition coefficients, CIT and DCIT were enriched by a factor of 25 to 30. In addition, sample clean-up occurred during LPME since salts, proteins and the majority of endogenic substances were unable to penetrate the hexyl ether layer. Since the extracts	Human Plasma	176

		were aqueous, they were injected directly into the CE instrument. Limits of quantification (S/N= 10) for CIT and DCIT in plasma were 16.5 ng/ml and 18 ng/ml respectively, while the limits of detection (S/N=3) were 5 ng/ml and 5.5 ng/ml respectively.		
5.	Micellar electrokinetic Capillary electrophoresis	A new determination procedure for these compounds (milnacipran, venlafaxine, desmethylvenlafaxine, mirtazapine, esmethylmirtazapine, citalopram, desmethylcitalopram, fluvoxamine, paroxetine, sertraline and fluoxetine) was developed by micellar electrokinetic capillary chromatography (MEKC) with diode array detection (DAD). Separation and determination were optimised on an uncoated fused-silica capillary (600 mm, 75 microm I.D.). The migration buffer consisted of 20 mM sodium borate, pH 8.55, with 20 mM SDS and 15% isopropanol, at an operating voltage of 25 kV. The column temperature was maintained at 40 degrees C. Injection in the capillary was performed in the hydrodynamic mode (0.5 p.s.i., 15 s). In these conditions, the migration time of the antidepressants was less than 11 min.	Blood Urine	177
6.	HPLC, column switching and fluorescence detector	High-performance liquid chromatography with a successive column-switching technique was developed for simultaneous determination of citalopram and its four metabolites in plasma. Plasma samples were injected directly, and the target compounds were purified and concentrated with an inexpensive commercial octadecyl guard column. Then, the six-port valve was switched, and the compounds retained in the column were eluted by the back-flush	Dog and Rat Plasma	178

		method using 20 mM phosphate buffer (pH 4.6)-acetonitrile (70:30, v/v) containing 0.1% diethylamine and separated with an ODS column. The compounds were assayed with a fluorescence detector at an excitation wavelength of 249 nm and an emission wavelength of 302 nm. At least 30 plasma samples could be treated with an octadecyl guard column. The limits of quantitation of this method were 2.0 ng/ml for citalopram, desmethylcitalopram, didesmethylcitalopram, citalopram propionic acid and citalopram N-oxide. This method was applied to a pharmacokinetic study in dogs and a toxicokinetic study in rats.		
7.	Capillary electrophoresis	Several CE methods have been developed to achieve the chiral separation of citalopram (CIT) and its metabolites demethylcitalopram (DCIT), didemethylcitalopram (DDCIT), and citalopram N-oxide (CIT-NO). All of these compounds were present as racemic mixtures. The best method, which led to the first ever chiral screening of CIT, DCIT, DDCIT, and CIT-NO, involved the use of carboxymethyl-gamma-CD (CM-gamma-CD) and the entangled polymer hydroxypropylmethylcellulose (HPMC) as chiral and selectivity additives, respectively, in the buffer system. In an effort to improve the selectivity and sensitivity of the method, the chemical and instrumental parameters were optimized. The best conditions were short-end anodic hydrodynamic injection (6 s, 0.7 psi); as BGE pH 5, 20 mM phosphate buffer, 0.2% w/v CM-gamma-CD, 0.05% w/v HPMC; voltage of 28 kV with a ramp	Urine	179

		applied (0.4 s); cartridge temperature of 20 degrees C; detection at 205 nm. In addition, a simple and rapid achiral CE method for the determination of citalopram propionic acid (CIT-PA, the only anionic metabolite of CIT) is also reported for the first time. Prior to the electrophoretic procedure it was necessary to apply an extraction and preconcentration step to obtain analytes from the human urine samples. This was achieved using an optimized SPE process. Moreover, an innovatory experimental and statistical design approach, which involves the simultaneous evaluation of the global robustness and ruggedness effects, was applied. Both of the proposed methods proved to be very useful in the chiral pharmacokinetic screening of CIT and related metabolites in clinical human urine samples.		
8.	Spectrophotometer	A high-performance liquid chromatographic method is used for the determination of citalopram [1-(3-dimethylaminopropyl)-1-(4-fluorophenyl)-5-phthalancarbonitrile+ ++] and four of its metabolites (the methylamino, amino, propionic acid and N-oxide derivatives) in plasma and urine. The plasma samples were extracted with diethyl ether at pH 10 and pH 4. Filtered urine samples could be injected directly on to the column.	Plasma and urine	180
9.	Fluorescence detector	A reliable and sensitive high-performance liquid chromatographic method for the determination of the recent antidepressant citalopram and two metabolites in human plasma has been developed. Fluorescence detection at 300 nm was used,	Human Plasma	181

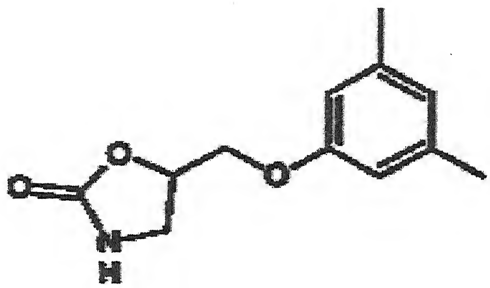
		exciting at 238 nm. Separation was obtained using a reversed-phase column (C18, 250 × 3.0 mm i.d., 5 µm) and a mobile phase. 40% acetonitrile: 60% aqueous tetramethylammonium perchlorate (pH 1.9). Calibration curves were linear over a working range: 5–300 ng mL−1 for citalopram, 2.5–150.0 ng mL−1 for desmethylcitalopram and 2.5–50.0 ng mL−1 for didesmethylcitalopram. The limits of quantitation (LOQ) were 1.5 ng mL−1 for citalopram and desmethylcitalopram and 2.0 ng mL−1 for didesmethylcitalopram.		
10.	Chiral Capillary Electrophoresis	A chiral capillary electrophoresis (CE) system allowing simultaneous enantiomer determination of citalopram (CIT) and its pharmacologically active metabolite desmethylcitalopram (DCIT) was developed. Excellent chiral separation was obtained using 1% sulfated-@b-cyclodextrin (S-@b-CD) as chiral selector in combination with 12% ACN in 25 mM phosphate pH 2.5. Samples were prepared by liquid-phase microextraction (LPME) based on a rodlike porous polypropylene hollow fibre. CIT and DCIT were extracted from 1 ml plasma made alkaline with NaOH, into dodecyl acetate impregnated in the pores of a hollow fibre, and into 20 mM phosphate pH 2.75, inside the hollow fibre. The acceptor solution was directly compatible with the CE system. Efficient sample clean-up was seen, and the recoveries were 46 and 29% for the enantiomers of CIT and DCIT, respectively, corresponding to 31 and 19 times enrichment. The limit of quantification (S/N=10) was <11.2 ng/ml, intra-day precision was <12.8% RSD, and inter-day	Human Plasma	182

		precision was <14.5% RSD, for all enantiomers. The validated method was successfully applied to simultaneous determination of enantiomer concentrations of CIT and DCIT in plasma samples and biological matrices.		

3.7 Metaxalone[183-186]

3.7.1 General Information

Structure:



Chemical Name : 5-[(3,5-dimethylphenoxy)methyl]oxazolidin-2-one

Molecular Formula : $C_{12}H_{15}NO_3$

Molecular Weight : 221.252 g/mol

Description : White crystalline powder.

Solubility : Freely soluble in chloroform, soluble in methanol and in 96% ethanol, but practically insoluble in ether or water.

Category : skeletal muscle relaxant.

3.7.2 Reported methods of analysis

No method was reported as per our knowledge, till date regarding quantitation of impurities of metaxalone.

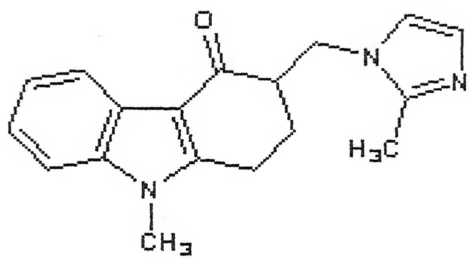
Sr.No	Method	Sailent Features	Matrix	Ref. No.
1.	LCMS	A simple, rapid, sensitive, and selective liquid chromatography–tandem mass spectrometry (MS) method was developed and validated for the quantification of metaxalone, a skeletal muscle relaxant, in human plasma using galantamine as internal standard (IS). Following liquid–liquid extraction, the analytes were separated using an isocratic mobile phase on a reverse phase C18 column and analyzed by MS in the multiple reaction monitoring mode using the respective [M+H]+ ions, m/z 222/161 for metaxalone and m/z 288/213 for the IS. The assay exhibited a linear dynamic range of 50–5000 µg/L for metaxalone in human plasma. The lower limit of quantification was 50 µg/L with a relative standard deviation of less than 10%. Acceptable precision and accuracy were obtained for concentrations over the standard curve range. A run time of 2.5 min for each sample made it possible to analyze more than 400 human plasma samples per day.	Human Plasma	187
2.	HPLC	Metaxalone was quantified by high-performance liquid chromatography (HPLC). The analytical column used was either a Waters NovaPak, C4 µm, 150 mm × 3.9 mm (Waters Corp, Milford, MA) or an Alltech Econosphere, 5 µm, 4.6 mm × 150 mm (Alltech Associates Inc, Deerfield, IL). The mobile phase consisted of 0.005M sodium dihydrogen phosphate buffer:methanol (420:580). A flow rate of 1 mL/minute resulted in a retention time of 3.5 minutes. The	Formul-ation	188

		peak area was recorded at 273 nm Samples of 5 mL were removed from the dissolution vessel (either Apparatus 2 or 3) and tested without further dilution.		

3.8 ONDANSETRON[189-191]

3.8.1 General Information

Structure:



Chemical Name : 9-methyl-3-[(2-methyl-1H-imidazol-1-yl)methyl]-1,2,3,9-tetrahydrocarbazol-4-one

Molecular Formula : $C_{18}H_{19}N_3O$

Molecular Weight : 293.363 g/mol

Description : White to off white powder.

Solubility : Sparingly soluble in water and in alcohol; very slightly soluble in acetone, in chloroform, and in ethyl acetate; slightly soluble in dichloromethane and in isopropyl alcohol; soluble in methyl alcohol.

Category : Antiemetics

3.8.2 Reported methods of analysis

Sr.No	**Method**	**Sailent Features**	**Matrix**	**Ref. No.**
1.	LCMS	Ondansetron and its hydroxylated metabolites were determined in human serum using solid-phase extraction (SPE) and liquid chromatography/positive ion electrospray tandem mass spectrometry. Pyrimethamine was used as the internal standard. The analytes were eluted from the SPE cartridge using 2 x 1 ml of methanol containing 0.5% triethylamine, evaporated under vacuum and the residue was reconstituted in the mobile phase. The liquid chromatographic separation was achieved on a silica column using a mobile phase of aqueous 20 mM ammonium acetate (pH 4.7)-acetonitrile (85 : 15, v/v) at a flow-rate of 0.4 ml min(-1). The method was linear over the range 1-500 ng ml(-1) for ondansetron and each of the metabolites in human serum. The intra-day accuracy was better than 9.1% and the precision was <10.3%; the inter-day accuracy was better than 9.5% and the precision was <12.6%. The limit of detection was 250 pg ml(-1) based on a signal-to-noise ratio of 3. The absolute recovery from serum for all analytes was >90%.	Human plasma	192
2.	HPTLC	Separation of impurity B and ondansetron by modifying the pharmacopial method ,the mobile phase employed is chloroform: ethyl acetate : methanol: ammonium hydroxide::18:5:4:0.15 v/v. silica plate choosen was 60WRF254s Densitometry mode at 311 nm was choosen.	Formulat ion	193

3.	HPLC and TLC	Imp D was analysed by Hplc using mobile phase 0.02Mmonobasic potassium phosphate (previously adjusted with 1Msodium hydroxide to a pH of 5.4)and acetonitrile (80:20). The liquid chromatograph is equipped with a 328-nm detector and a 4.6-mm ×20-cm column that contains packing L10.The flow rate is about 1.5mLper minute. Impurity A and B were analysed by TLC, chromatogram is developed in a solvent system consisting of a mixture of chloroform, ethyl acetate, methanol, and ammonium hydroxide (90:50:40:1).Impurity C was analysed by HPLC, 0.02Mmonobasic sodium phosphate (previously adjusted with 1Msodium hydroxide to a pH of 5.4)and acetonitrile (50:50). The liquid chromatograph is equipped with a 216-nm detector and a 4.6-mm ×20-cm column that contains packing L10.The flow rate is about 1.5mLper minute.	Bulk Drug	194
4.	HPLC	This study investigate the potential utilization of metal oxide-based stationary phases in analytical evaluation of ondansetron and its five pharmacopoeial impurities. The commercially available ZrO(2)-based columns (e.g. Zr-PBD, Zr-PS, Zr-C18) and TiO(2)-based column were used. The effect of an organic modifier (type and ratio), a buffer (type, pH and concentration) and the influence of temperature was investigated. The separation of ondansetron and its five pharmacopoeial impurities was successfully accomplished on a Zirchrom((R))-PBD column using a mobile phase consisting of acetonitrile-ammonium phosphate (25mM, pH 7.0) (18:82, v/v). Detection was performed at 216nm and the analysis was completed within 7.5min. The paper proves	Bulk drugs	195

		metal oxide-based stationary phases as an alternative to classical silica-based stationary phases in pharmaceutical analysis.		
5.	TLC and HPLC	Impurity B is analysed by TLC using mobile phase concentrated ammonia R, methanol, ethyl acetate, methylene chloride (2:40:50:90 V/V/V/V). TLC silica gel F254 plate R is used and examined in ultraviolet light at 254 nm. HPLC analysis is done for imp A, C,D,E,F,G,H. Mobile phase is a mixture of 20 volumes of acetonitrile and 80 volumes of a 2.8 g/l solution of sodium dihydrogen phosphate monohydrate previously adjusted to pH 5.4 with a 40 g/l solution of sodium hydroxide. Flow rate at 1.5 ml/min. and detector at 216 nm. column used is spherical nitrile silica gel for chromatography R (5 µm) with a specific surface area of 220 m2/g and a pore size of 8 nm. Relative retentions with reference to ondansetron (retention time = about 18 min): impurity E = about 0.1; impurity F = about 0.2; impurity C = about 0.4; impurity D = about 0.5; impurity H = about 0.7; impurity A = about 0.8; impurity G = about 0.9.	Bulk drugs	196

3.9 RESEARCH ENVISAGED

Unlike any other commodity, the manufactured drug and its formulations must strictly comply with standards and regulations set forth by the regulatory authorities. The efficacy and safety of medicinal products can only be assured by analytical monitoring of its quality. Hence, the quality control laboratory forms the heart of drug industry. It is here that various procedures are required for analysis of existing drug and formulations.

In recent times, rational approaches propounded by various pharmacologists and synthetic organic chemists have resulted in development of newer and newer chemical entities, and hence the analytical chemist has to accept the challenge of developing reliable methods for analysis of such newly developed molecules, control the extent of related substances, impurities, degradation products and various other contaminants.

With the increasing importance of quality functions, various regulatory authorities are becoming more and more demanding. It is not only essential to ensure the presence of required amount of the drug in the bulk or formulation throughout its shelf life, but also to control the extent of related substances, degradation products etc. This is because some of the degradation products may be toxic, may have undesirable adverse pharmacological effect or may alter the bioavailability of the drug. Acceptable limit of such impurities is very less, to the extent of 0.1 % or less. Decrease in assay value by 5.0 % in accelerated stability testing is considered to be significant. Hence it is very necessary to have analytical technique which can detect decrease in assay and increase in impurities. Also the method should be efficient and reproducible.

Although there are many formulations in market, there are very few official methods for the analysis of active ingredients of these formulations. Although official method exists, it may suffer the lack of specificity and may not be stability indicating. Hence, the proposed work was undertaken with an aim to develop new methods that will serve adequately for routine drug analysis in pharmaceutical industries. The compilation of above scientific work shall help the national drug industry by providing methods for routine analysis.

In the present work, emphasis was put on to develop simple, accurate, precise and rapid methods for estimation of the active ingredients or impurities in the selected formulations or bulk drug using HPLC.

Literature survey clearly reveals that no efficient analytical method is available for assay of formulations containing candesartan, captopril, propranolol, Terazosin, Verapamil, and impurity profile of citalopram, metaxalone, ondansetron. Although a number of methods have been reported for the analysis of active ingredients in the selected formulations, these methods can not be adopted as a routine quality control test, due to one or more reasons. Hence, the proposed work was undertaken with an aim to develop new stability indicating methods that will serve adequately for routine drug analysis in pharmaceutical industries.

3.10 PLAN OF WORK

The HPLC work was planned as:

- Review of all literature available on the analysis of drugs in single and multi component formulations.
- Critical examination of the structure of the drugs and their physicochemical properties, such as solubility, dissociation constant etc. This would help in selecting the chromatographic parameters such as column, mobile phase, and detector.
- Selection of a method for quantitative chromatographic analysis.
- Development of method.
- Validation of method.
- Statistical evaluation of the results of analysis.
- Documenting the method.

CHAPTER – 4

EXPERIMENTAL

The preliminary investigation done regarding general information and reported method of analysis available helped in development of simple, reliable and stability indicating methods. The main aim of the development was to set up chromatographic system capable of separating main drug, placebo and degradation products for the assay development of formulations under study and separation of main drug, impurities and degradation products for the impurity profile study of bulk drugs.

The development of method for each drug is done by understanding and solving following questions.[197]

1. What sort of answers are required.....

a) Is the method to be analytical or preparative.

b) If Analytical, quantitative or qualitative.

c) High throughput or maximum detail

d) To what level assay must be validated or impurities must be determined.

e) If any previous literature available for separation of compound.

2. Study the compound, its chemical structure and behaviour.

a) What is the molecular weight.

b) Does it isomerise or already exist in different isomeric forms

c) In what it is soluble.

d) What column/eluent will be appropriate.

e) what kind of sample preparation is required.

To develop a suitable method most of the questions are needed to be answered.

4.1 DEVELOPED ANALYTICAL METHOD

4.1.1 Candesartan

Candesartan is a non pharmacopeial drug and literature survey reveals that there was no cost effective method reported as per our knowledge which can be used on regular basis for quantification of candesartan, most of the reported method involves drugs in combinations or plasma matrix.

we had used previously reported data such as Pka value of Candesartan which is 5.3[198], for which buffer choice of KH_2PO_4 is taken as it gives basic pH, helps in ionization of the drug and various trials of organic phase methanol were tried to get desired retention time, so that all degradation products are well separated and peak shape of Candesartan has good asymmetry.

The procedure mentioned under reference no 117 suggests use of short C18 columns (4.6mm x 15 cm), where it was observed that it creates back pressure and resolution problem, as in stability samples lot of degradation peaks are generated and large number of samples are to be analysed, A thermohypersil C18 column, 4.6 mm x 25.0cm, 5 µ is selected as it resolved degradation peaks and proves to have more column life for stability samples.

Candesartan drug spectra shows to have lambda max at 238 nm but the degradation peaks shows high response at 220 nm so this wavelength proved to be ideal for stability indicating method.

Various trials are undertaken to achieve good resolution, peak shape and proper retention time(about 11.3 min) for candesartan peak, of about concentration of 500 ppm in mobile phase. Final conditions achieved for method are tabulated below.

Table 4.1.1 : Chromatographic conditions for Candesartan tablet.

S.No	Parameters	Description/Value
1.	Mobile Phase	buffer(6.8 g KH_2PO_4 /1000ml water): methanol : : (40:60)
2.	Flow rate	1.0 ml/min
3.	Detector	220 nm
4.	Column	Thermo-hypersil,C18,250 * 4.6 mm,5 µm
5.	Injection volume	20 µl
6.	Run time	30 min

4.1.2 Captopril

Captopril is a pharmacopeial drug. USP suggests titremetry method for captopril tablets, which is not a stability indicating method as degradation study cannot be performed successfully, whereas in BP the HPLC method for captopril tablets shows asymmetry of more than 2.0 and retention time of 3.0 min which is very early, either with or very close to the solvent front, this renders the quantitation of Captopril tablets very difficult. Our effort to modify the procedure by changing ratio of organic phase in mobile phase and column temperature did not improve the results.

The previous procedures mention uses of C18 columns(Reference no 123 and 125). In order to increase the retention time of Captopril peak, we switched to trials with C8 column, as it reduces hydrophobicity of the column, which in turn increase retention of protonated Captopril , but still the peak shape has not improved, showing high asymmetry, so to reduce it, trials with ion –pairing reagent were undertaken. Methane sulphonic acid was added in mobile phase as it helped in reducing the asymmetry of

the Captopril peak, higher alkyl chain of sulphonic acid is avoided as it may increase the retention time of Captopril peak, as we were comfortable with retention time by choosing Luna C8 column.

Various trials are undertaken to smoothen the desired method, the final conditions achieved are tabulated as under.

Table 4.1.2 : Chromatographic conditions for Captopril tablet.

S.No	Parameters	Description/Value
1.	Mobile Phase	water : acetonitrile: tetrahydrofuran :methane sulfonic acid :: 80:10:10:0.1
2.	Flow rate	1.0 ml/min
3.	Detector	220 nm
4.	Column	Luna,C8,250 * 4.6 mm,5 µm
5.	Injection volume	20 µl
6.	Run time	30 min

4.1.3 Propranolol

Propranolol is a pharmacopeial drug. B.P suggest a spectrophotometric method which is unsuitable for stability studies as degradation study cannot be performed, where as U.S.P suggests a HPLC method using mobile phase containing Sodium Dodecyl sulphate(2.0 gm/litre requirement as per reference no.137) which is a costly reagent approximately 100 gms costs 21$, hence it is not a cost effective method specially for the analysis of stability samples where large number of samples are to be analysed on regular basis.

The structure of propranolol reveals that it exhibits zwitterion having amine and alcohol group in a compound on different atoms , hence we tried to replace anionic

ion pairing reagent sodium dodecyl sulphate of mobile phase by cationic ion pairing reagent triethyl amine, so that molecule can be ionized, previously reported data suggests Pka value of propranolol to be 9.5[199],so a acidic pH of 4.0 is acheived by adjusting pH of mobile phase by formic acid.

Various trials of organic phase acetonitrile is tried to get desired retention time, so that all degradation products are well separated and peak shape of propranolol has good asymmetry.

Propranolol has dual lambda max in u.v region ,at 225 and 290 nm[200], as the mobile phase selected has cutoff value below 200 nm, hence lower wavelength (225 nm) was selected to detect large number of impurities.

Final conditions achieved for method are tabulated below.

Table 4.1.3 : Chromatographic conditions for Propranolol tablet.

S.No	Parameters	Description/Value
1.	Mobile Phase	Buffer(5.0 g Triethylamine /1000ml water pH 4.0 by HCOOH): acetonitrile::70:30.
2.	Flow rate	1.5 ml/min
3.	Detector	225 nm
4.	Column	Phenomenex,Luna,C18,250 * 4.6 mm, 5 µm
5.	Injection volume	20 µl
6.	Run time	30 min

4.1.4 Terazosin

Terazosin is official in USP (Reference No.147), the mobile phase uses concentrated citrate buffer (12.0 gm sodium citrate.2 H_2O + 28.5 gm Citric acid) which degenerates the C8 column used very rapidly, the system suitability

requirement are also very stringent (theoretical plates NLT 12000,Tailing factor NLT 0.9 and NMT 1.3)which made it difficult to analyse large number of stability samples in same column, hence a method which is more convienent to handle stability sample is developed.

The Pka value of terazosin is 7.1[201] being a neutral molecule acidic or basic pH can be used to ionize the molecule, a simple buffer of KH_2PO_4 is choosen, various trials of organic phases were tried, but methanol is found to be best suitable, C18 column is more rugged and has more life time which is found to be suitable for the analysis of terazosin, Lambda max of terazosin is 245 nm [202] hence it is choosen.

Final conditions achieved for method are tabulated below.

Table 4.1.4 : Chromatographic conditions for Terazosin tablet.

S.No	Parameters	Description/Value
1.	Mobile Phase	Buffer(6.8 g KH_2PO_4 /1000ml): Methanol::60:40.
2.	Flow rate	1.0 ml/min
3.	Detector	245 nm
4.	Column	Thermohypersil,C18,250 * 4.6 mm,5 µm
5.	Injection volume	20 µl
6.	Run time	20 min.

4.1.5 Verapamil

Verapamil is pharmacopeial drug, The assay method mentioned in B.P is a spectrophotometric, where as USP suggests a HPLC method , which has few drawbacks like uses of short column creates problem of back pressure and resolution in stability samples,separation problems of degradation peaks etc. Hence to

overcome the problem for estimation of Verapamil in stability samples where large numbers of sample is to be quantified on regular basis a simple reliable method is developed to remove the drawbacks of USP method, Verapamil is a basic drug a neutral pH of 7.0 is selected for which buffer choice of Na_2HPO_4 is taken and various trials of organic phase acetonitrile is tried to get desired retention time, so that all degradation products are well separated and peak shape of verapamil has good asymmetry. The previous procedures mention uses of short C18 columns 4.6mm x 12.5 – 15 cm, where it is observed that it creates back pressure and resolution problem as in stability samples lot of impurities are generated and large number of samples are to be analysed , hence a long column of 4.6 mm x 25.0cm is selected. detector wavelength is selected at 232 nm as verapamil has dual lambda max in u.v region ,at 232 and 278 nm, as the mobile phase selected has cutoff value below 200 nm, hence lower wavelength is selected to detect large number of impurities.

Various trials are undertaken to smoothen the desired method, the final conditions achieved are tabulated as under.

Table 4.1.5 : Chromatographic conditions for Verapamil tablet.

S.No	Parameters	Description/Value
1.	Mobile Phase	Buffer(1.4 g Na_2HPO_4 /1000ml, pH 7.0 by H_3PO_4): Acetonitrile::50:50.
2.	Flow rate	2.0 ml/min
3.	Detector	232 nm
4.	Column	Thermohypersil,C18,250 * 4.6 mm,5 µm
5.	Injection volume	20 µl
6.	Run time	30 min.

4.1.6 Citalopram

Citalopram is a non pharmacopeial drug and references suggests that degradation study and impurity profiling is not much studied, we had describes the development and validation of a stability indicating isocratic reversed-phase HPLC method for simultaneous determination of seven related impurities in presence of their degradation products as per ICH guidelines for citalopram bulk drug. The impurities are 1-(3-Dimethylaminopropyl)-1-(4-fluorophenyl)-1,3 dihydro-isobenzofurn-5-carboxylic acid amide.(impurity-1), 1-(3-Dimethylaminopropyl,N oxide)-1-(4-fluorophenyl)-5-phthalan carbonitrile.(impurity-2), 1-(3-methylaminopropyl)-1-(4-fluorophenyl)-5-cyanophthalan oxalate.(impurity-3), 1-(3-dimethylaminopropyl)-1-(4-fluorophenyl)- phthalan oxalate.(impurity-4), 1-(3-dimethylaminopropyl)-1-(phenyl)- 5-phthalan carbonitrile oxalate.(impurity-5), 1-(3-dimethylaminopropyl)-1-(4-fluorophenyl)- 5-chlorophthalan oxalate.(impurity-6), 1-(3-dimethylaminopropyl)-1-(4-fluorophenyl)- 5-bromophthalan oxalate.(impurity-7).

The functional group of impurities 1, 2, 3 and 4 suggests basic pKa value and impurities 5,6 and 7 had mild acidic pKa value, hence to ionized them completely it is necessary to have a mobile phase having acidic or neutral pH. For acidic pH a phosphate buffer ($(NH_4)_2HPO_4$) is choosen, Initially pH of the mobile phase was kept at 7.0 and it was observed that basic nature impurities are eluting very closely hence a slightly modified acidic pH 6.8 was choosen which had improved resolution between peaks. Triethylamine and sodium salt of hexane sulphonic acid was used as a modifier to rectify tailing problem of basic and acidic impurities peaks respectively. To develop a suitable and precise LC method for the determination of Citalopram and its related impurities, different combinations of acetonitrile, methanol and buffer ratio

were tried. Different brands of columns are observed. to achieve the minimum analysis time, the best results were obtained from C18, 250*4.6mm,5µ , Restek (Pinnacle –II,U.S.A) column. The temperature of the column was kept at 40°C as it helps in reducing analysis time by fast elution of impurity 5, 6 and 7. By keeping all the above parameters, the mobile phase and instrumental conditions is developed which separates all the impurities, degradation products and Citalopram in a short runtime of 30 min, eluting the first peak at 3.4 min and last at 24.5 min.

Final conditions achieved for method are tabulated below.

Table 4.1.6 : Chromatographic conditions for Citalopram Bulk drug.

S.No	Parameters	Description/Value
1.	Mobile Phase	buffer (1.3 gm diammonium hydrogen orthophosphate in 1000ml water + 2ml of triethylamine, pH 6.8 by orthophosphoric acid): Methanol: Acetonitrile::45: 45:10 .+0.94 g of sodium hexane sulphonic acid
2.	Flow rate	2.0 ml/min
3.	Detector	220 nm
4.	Column	Restek C18, 250*4.6mm, 5µ
5.	Injection volume	20 µl
6.	Run time	30 min.
7.	Column Temperature	40°C.

4.1.7 Metaxalone

Metaxalone is a non pharmacopeial drug and literature survey shows that there is no detail available about the degradation and related substance of metaxalone. A stability–indicating, rapid and reliable HPLC method was developed for the

quantitative determination of metaxalone and its related impurities 3-(3,5-Dimethylphenoxy)-2-hydroxypropylamine hydrochloride (imp-1),Ethyl [3-(3,5-dimethylphenoxy)-2-hydroxypropyl] carbamate (imp-2),3-[3-(3,5-dimethylphenoxy)-2-hydroxypropyl]-5-(3,5-methylphenoxy)methyl-oxazolidine-2-one (imp-3), Ethyl N,N-bis[3-(3,5-dimethylphenoxy)-2-hydroxypropyl] carbamate (imp-4) and Ethyl N,N-bis[3-(3,5-dimethylphenoxy)-2-hydroxypropyl] amine (imp-5).

There was no method reported regarding the separation of related impurities of metaxalone. A study shows that at pH 4.5 the solubility of metaxalone is highest[203].The functional group of all the impurities shows pKa value on basic side, hence to ionized them completely it is necessary to have a mobile phase having acidic pH. For acidic pH a phosphate buffer (KH_2PO_4) is choosen, To develop a suitable and precise LC method for the determination of metaxalone and its related impurities, different mobile phase, columns different pH is applied. Initially mobile phase is kept at a pH value of 4.5 as metaxalone had highest solubility and C18 column but a pronounced tailing of peaks is observed. This is because , at pH values above 3.5 silonols present in the column acts as a weak acids,which will have strong interactions with compounds having basic or amino groups. These interaction results in a dual retention process that produces peak tailing. Pronounced peak tailing makes quantitation more difficult and less accurate hence mobile phase having pH value of 3.0 and C 8 column was selected to overcome this problem. The organic phase composition is kept at 50 %v/v to buffer as it is required to separate five impurities and metaxalone in short time as possible, with good separation and avoiding dead volume complications in the method. By keeping all the above parameters, the mobile phase and instrumental conditions is developed which

separates all the impurities and metaxalone in a runtime of 40 min, eluting the first peak at 3.2 min and last at 37.0 min. The resolution obtained between two nearest peak is 4.75.

Final conditions achieved for method are tabulated below.

Table 4.1.7 : Chromatographic conditions for Metaxalone Bulk drug.

S.No	Parameters	Description/Value
1.	Mobile Phase	buffer (6.0 gm KH_2PO_4in 1000ml water pH 3.0 by H_3PO_4): Acetonitrile::50:50 .
2.	Flow rate	1.0 ml/min
3.	Detector	225 nm
4.	Column	Hypersil C8, BDS, 250*4.6mm, 5µ
5.	Injection volume	20 µl
6.	Run time	40 min.
7.	Column Temperature	ambient.

4.1.8 Ondansetron

Ondansetron is a pharmacopeial drug. The USP 28 monograph suggests four impurities A, B , C and D for ondansetron drug. TLC method is suggested for imp A, B, and C, where as a limit test for impurity D by HPLC. In our study we tried to develop a single method for the estimation of impurity A ,D and C. Impurity B had a lambda max at 305nm, hence in the method developed it is not possible to detect impurity B as the detector wavelength choosen is 216 nm. Impurity A is 3[(dimethylamino)methyl}-1,2,3,9-tetrahydro-9-methyl-4H-carbazol-4-one hydrochloride, impurity C is 1,2,3,9-Tetrahydro-9-methyl-4H-carbazol-4-one and impurity D is 1,2,3,9-Tetrahydro-9-methyl-3methylene-4H-carbazol-4-one.All

impurities of ondansetron had amine group suggesting a basic PKa value, hence to ionize them a acidic mobile phase is choosen.KH_2PO_4 buffer having a pH value of 5.4 is selected, Triethyl amine is added as it enhances the sharpness of basic molecules and increases the resolution. Detector wavelength is set at 216 nm as it is found to be more suitable to detect all the impurities and degradation products. Impurity A contains two tertiary amine is eluted first, followed by impurity C having one tertiary amine and lastly impurity D was eluted.

Final conditions achieved for method are tabulated below.

Table 4.1.8 : Chromatographic conditions for Ondansetron Bulk drug.

S.No	Parameters	Description/Value
1.	Mobile Phase	buffer (2.7 gm KH_2PO_4in 1000ml water + 5.0 ml of triethyl amine pH 3.0 by H_3PO_4): Acetonitrile::70:30 .
2.	Flow rate	1.2 ml/min
3.	Detector	216 nm
4.	Column	Luna,C8,250*4.6mm, 5µ
5.	Injection volume	20 µl
6.	Run time	30 min.
7.	Column Temperature	ambient.

4.2 VALIDATION

Validation is a logical process that is conducted in parallel with method development. If a method that is being developed will ever be used in earnest, then it must be validated. The data obtained during method development may be used towards validation and the outcome of validations may force changes in method.(which must

be revalidated),This iterative procedure is known as the "development/validation cycle"[204]

Validations of analytical methods and procedures in a quality control (Q.C) laboratories is implemented mainly at the time of transfer or introduction of the method developed by analytical development lab within the group companies or elsewhere. However it is sometime necessary to develop a new or improved method of analysis for Q.C laboratories own use[205].The most important factor for selection of the analytical techniques and instrument is the objective of the analysis. The method must meet the requirement of guidelines issued by the regulatory authorities where the method is intended for compliance purpose. Features of the sample and availability of reference material are also key factors in determining an analytical procedure and detection method, which then relate to various analytical validation characteristics and parameters. Cost effectiveness is a pressing facts even in Q.C Labs. While at the same time quality standards must be kept at high as necessary. Time and man power saving methods must be developed and applied in routine operations in the Q.C lab of the pharmaceutical company.

HPLC VERSUS OTHER ANALYTICAL TECHNIQUES[206]

The HPLC method is now days, the most widely used versatile method in pharmaceutical area. HPLC analytical validation is a complex procedure compare to relatively simple techniques such as volumetric analysis, spectrophotometric detection but HPLC had superiority of achieving the important factor of validation "specificity". Below are few drawbacks of some commonly used techniques are mentioned.

Volumetric analysis:

Volumetric analysis using titration methods are very cheap and simple ,specially when sophisticated instruments like auto-titrator are used. Validation parameters such as precision, accuracy, LOD, LOQ and robustness can be achieved, stability of solution is independent of technique of analysis, but the main fallout is the test for "specificity". It is particularly important when it is to determine the purity of complex molecules like drugs. Drugs are generally prepared by a multi stage synthesis and contains water, solvents, trace catalyst, inorganic and structurally related synthetic by products i.e intermediates, isomers, degradation products and product from side reactions. For Example a basic compound it is highly likely that all or almost all the structurally related impurities will also be basic therefore titration against an acid would not give purity but would give some measure of the total amount of drug, structurally related impurities and other basic impurities. which is certainly unacceptable as it is most important to determine the structurally related impurity since it is these ,that are most likely to give rise to toxicological problems.

U.V. Spectrophotometery :

Similar difficulties arise when using U.V Spectrophotometry in which analyte concentration are determined by measuring the absorbance of U.V light by the sample solution. For structurally related impurities of a uv absorbing compound it is highly likely that they will contain a very similar chromophore therefore incident radiation of any particular wavelength of uv spectrum of the compound would almost certainly be absorbed by structurally related compounds as well as the compound itself.

he most common approach to solve the critical problem of lack of specificity, is to ıeasure physical or chemical property of the analyte that is unique i.e the mass pectrum of the compound (the pattern obtained when a compound is ionized under ıigh energy condition and the resultant ion traverse a magnetic field) which gives :haracteristic information on molecular mass and the mass of fragments of the nolecule. However mass spectrometry requires expensive instrumentation and it vould be in appropriate option for routine quantitative analysis(also it fails to distinguish between isomers.)

HPLC today plays a dominant role in providing methods for pharmaceutical analysis. It is the most versatile technique for quickly analyzing large numbers of different samples: checking purity in the development of drugs and quality assurance in the process of quality control of final products.

4.2.1 Candesartan

The following solutions are required to be prepared for routine analysis of calculation of assay of candesartan tablet.

System suitability/Standard solution

Transfer about 50 mg of candesartan standard, accurately weighed, to a 100 ml volumetric flask. Dissolve in and dilute upto mark with mobile phase. Transfer 10 ml of the above solution to 100 ml and make up with mobile phase.

Sample preparation

Transfer blend of candesartan tablets equivalent to 5 mg of candesartan to a 100 ml volumetric flask. Add about 20 ml of mobile phase and sonicate for 5 minutes with

intermediate shaking and dilute with mobile phase to volume and mix. Centrifuge this solution at about 2000 RPM for 10 min and use upper clear solution for injection.

Calculations

$$\%\text{ Assay of candesartan} = \frac{AT}{AS} \times \frac{WS}{WT} \times D \times A \times \frac{P}{L}$$

Where :

AT = Average area count of candesartan peak in sample preparation.

AS = Average area count of candesartan peak in standard preparation.

WT = Weight of candesartan sample in mg.

WS = Weight of candesartan standard in mg.

D = Dilution factor

P = % purity of candesartan standard (as is basis)

A = Average Wt of candesartan tablets in mg

L = Label claim in mg

Validation

Following solutions were prepared for validation of method.

1. Identification

50.2 mg candesartan standard was weighed accurately, in a 100 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase. Transfer 10 ml of the above solution to 100 ml and make up with mobile phase.

2. System suitability/System Precision

50.2 mg candesartan standard was weighed accurately, in a 100 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase. Transfer 10 ml of the above solution to 100 ml and make up with mobile phase. This solution was injected six times, mean and RSD of detector response of candesartan was calculated.

3. Specificity of the method

No interference from excipients

This was demonstrated by preparing a placebo containing all excipients and injecting a sample prepared from the same. There are no peaks at the retention time of candesartan, thereby indicating that there is no interference from excipients.

Standard preparation

53.5 mg of candesartan standard was weighed accurately and transferred to 100 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase. Transfer 10 ml of the above solution to 100 ml and make up with mobile phase.

Table 4.2.1a : Chromatographic conditions for Degradation study.

Degradation	Condition
Acid degradation	67.33 mg candesartan tablet, and 2 ml 2M HCl. Heated at 70°C for 60 min.
Alkali degradation	67.23 mg candesartan tablet,and 2ml 2M NaOH. Heated at 70°C for 60 min.
Peroxide degradation	64.13 mg candesartan tablet, and 1 ml of 30.0% H_2O_2. Heated at 70°C for 60 min.
Sun-Light exposure	65.20 mg candesartan tablet kept for 2 hrs. in Sun-light.
Thermal degradation	65.94 mg candesartan tablet and 20ml mobile phase. Heated at 70°C for 60 min.

4. Linearity Study

The linearity of detector (UV) response for candesartan standard was determined by preparing and injecting solutions in the concentration range of 25 – 75 μg/ml (50-150 % of assay conc.) of candesartan standard.

Stock Solution

125.2 mg of candesartan standard was weighed accurately and transferred to a 50 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min volume was made with mobile phase. This solution was diluted as shown in Table - 4.2.1b

Table 4.2.1b : Solution preparation for linearity study of candesartan tablets

% level of std	Vol. of stock sol (ml)	Final Dil. (ml)	Final conc. Of candesartan (μg/ml)
50	1.0	100	25.04
75	1.5	100	37.56
100	2.0	100	50.08
125	2.5	100	62.60
150	3.0	100	75.12

5. Method Precision

Standard preparation

50.2 mg of candesartan standard was weighed accurately and transferred to 100 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase mixed thoroughly. Transfer 10 ml of the above solution to 100 ml and make up with mobile phase.

Sample preparation

Six sets of candesartan tablets of same lot were prepared as per the method on same day for analysis by the HPLC method. Table 4.2.1c shows solution preparation for method precision sets.

Table 4.2.1c: Solution preparation for method precision sets of candesartan Tablets

Set no.	Weight of sample(mg)	Final Dilution (ml)
1	63.36	100
2	62.62	100
3	63.14	100
4	63.35	100
5	63.34	100
6	63.22	100

Note: Average weight of 16 mg(content/tablet) of candesartan was calculated to be 201.6 mg (average wt of 10 tablets were observed).

6. Recovery(Accuracy)

Recovery study was performed by spiking candesartan standard in the placebo at levels 80%, 100% & 120 % of assay concentration in triplicate. The samples were chromatographed according to the assay procedure. Table 4.2.1d shows solution preparation for recovery study.

Table 4.2.1d : Solution preparation for recovery study of candesartan Tablets

Solution detail	Placebo wt(mg)	Volume of stock solution (ml) (linearity study)	Final Dilution (ml)
standard	-	2.0	100
80 %	185.6	1.6	100
100%	185.3	2.0	100
120%	185.9	2.4	100

7. Ruggedness

Method ruggedness was established by analysing sample at normal operating condition and at changed conditions then results were compared. Table no.4.2.1e shows details of solution preparation for ruggedness study.

Table 4.2.1e : Solution preparation for ruggedness study of candesartan Tablets

Parameters	Solution	Weight (mg)	Final dilution (ml)		
Original	Standard	50.20	100	10	100
	Sample	63.36	100	-	-
Changed	Standard	53.50	100	10	100
	Sample	67.70	100	-	-

8. Stability of analytical solution

System precision study solution was injected in duplicate at different time intervals and peak areas were recorded.

4.2.2 Captopril

The following solutions are required to be prepared for routine analysis of calculation of assay of captopril tablet.

System suitability/Standard solution

Transfer about 50 mg of captopril standard, accurately weighed, to a 100 ml volumetric flask. Dissolve in and dilute upto mark with mobile phase. Transfer 10 ml of the above solution to 100 ml and make up with mobile phase.

Sample preparation

Transfer blend of captopril tablets equivalent to 5 mg of captopril to a 100 ml volumetric flask. Add about 20 ml of mobile phase and sonicate for 5 minutes with

intermediate shaking and dilute with mobile phase to volume and mix. Centrifuge this solution at about 2000 RPM for 10 min. and use upper clear solution for injection.

Calculations

$$\% \text{ Assay of captopril} = \frac{AT}{AS} \times \frac{WS}{WT} \times D \times A \times \frac{P}{L}$$

Where :

AT = Average area count of captopril peak in sample preparation.

AS = Average area count of captopril peak in standard preparation.

WT = Weight of captopril sample in mg.

WS = Weight of captopril standard in mg.

D = Dilution factor

P = % purity of captopril standard (as is basis)

A = Average Wt of captopril tablets in mg

L = Label claim in mg

Validation

Following solutions were prepared for validation of method.

1. Identification

50.2 mg Captopril standard was weighed accurately, in a 100 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase. Transfer 10 ml of the above solution to 100 ml and make up with mobile phase.

2. System suitability/System Precision

50.2 mg captopril standard was weighed accurately, in a 100 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase. Transfer 10 ml of the above solution to 100 ml and make up with mobile phase. This solution was injected six times, mean and RSD of detector response of captopril was calculated.

3. Specificity of the method

No interference from excipients

This was demonstrated by preparing a placebo containing all excipients and injecting a sample prepared from the same. There are no peaks at the retention time of captopril, thereby indicating that there is no interference from excipients.

Standard preparation

50.3 mg of captopril standard was weighed accurately and transferred to 100 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase. Transfer 10 ml of the above solution to 100 ml and make up with mobile phase.

Table 4.2.2a : Chromatographic conditions for Degradation study.

Degradation	Condition
Acid degradation	63.2 mg captopril tablet, and 2 ml 2M HCl. Heated at 70°C for 60 min.
Alkali degradation	63.1 mg captopril tablet,and 2ml 2M NaOH. Heated at 70°C for 60 min.
Peroxide degradation	60.2 mg captopril tablet, and 1 ml of 30.0% H_2O_2. Heated at 70°C for 60 min.
Sun-Light exposure	61.2 mg captopril tablet kept for 2 hrs. in Sun-light.
Thermal degradation	61.9 mg captopril tablet and 20ml mobile phase. Heated at 70°C for 60 min.

4. Linearity Study

The linearity of detector (UV) response for captopril standard was determined by preparing and injecting solutions in the concentration range of 25 – 75 μg/ml (50-150 % of assay conc.) of captopril standard.

Stock Solution

125.6 mg of captopril standard was weighed accurately and transferred to a 50 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min. volume was made with mobile phase. This solution was diluted as shown in Table - 4.2.2b

Table 4.2.2b : Solution preparation for linearity study of captopril tablets

% level of standard	Vol. of stock solution (ml)	Final Dilution (ml)	Final concentrations of captopril (μg/ml)
50	1.0	100	25.12
75	1.5	100	37.68
100	2.0	100	50.24
125	2.5	100	62.80
150	3.0	100	75.36

5. Method Precision

Standard preparation

50.2 mg of captopril standard was weighed accurately and transferred to 100 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase mixed thoroughly. Transfer 10 ml of the above solution to 100 ml and make up with mobile phase.

Sample preparation

Six sets of captopril tablets of same lot were prepared as per the method on same day for analysis by the HPLC method . Table 4.2.2c shows solution preparation for method precision sets.

Table 4.2.2c : Solution preparation for method precision sets of captopril Tablets

Set no.	Weight of sample(mg)	Final Dilution (ml)
1	63.0	100
2	62.5	100
3	63.6	100
4	63.1	100
5	63.0	100
6	63.2	100

Note: Average weight of 25 mg(content/tablet) of captopril was calculated to be 315.0 mg (average wt of 10 tablets were observed).

6. Recovery(Accuracy)

Recovery study was performed by spiking captopril standard in the placebo at levels 80%, 100% & 120 % of assay concentration in triplicate. The samples were chromatographed according to the assay procedure. Table 4.2.2d shows solution preparation for recovery study.

Table 4.2.2d : Solution preparation for recovery study of captopril Tablets

Solution detail	Placebo wt(mg)	Volume of stock solution (ml) (linearity study)	Final Dilution(ml)
standard	-	2.0	100
80 %	290.0	1.6	100
100%	290.5	2.0	100
120%	292.1	2.4	100

7. Ruggedness

Method ruggedness was established by analysing sample at normal operating condition and at changed conditions then results were compared. Table no.4.2.2e shows details of solution preparation for ruggedness study.

Table 4.2.2e : Solution preparation for ruggedness study of captopril Tablet

Parameters	Solution	Weight (mg)	Final dilution (ml)		
Original	Standard	50.2	100	10	100
	Sample	63.0	100	-	-
Changed	Standard	50.3	100	10	100
	Sample	63.2	100	-	-

8. Stability of analytical solution

System precision study solution was injected in duplicate at different time intervals and peak areas were recorded.

4.2.3 Propranolol

The following solutions are required to be prepared for routine analysis of calculation of assay of Propranolol tablet.

System suitability/Standard solution

Transfer about 50 mg of Propranolol standard, accurately weighed, to a 50 ml volumetric flask. Dissolve in and dilute up to mark with mobile phase. Transfer 10 ml of the above solution to 100 ml and make up with mobile phase.

Sample preparation

Transfer blend of Propranolol tablets equivalent to 10 mg of Propranolol to a 100 ml volumetric flask. Add about 20 ml of mobile phase and sonicate for 5 minutes with

intermediate shaking and dilute with mobile phase to volume and mix. Centrifuge this solution at about 2000 RPM for 10 min. and use upper clear solution for injection.

Calculations

$$\% \text{ Assay of Propranolol} = \frac{AT}{AS} \times \frac{WS}{WT} \times D \times A \times \frac{P}{L}$$

Where :

AT	=	Average area count of Propranolol peak in sample preparation.
AS	=	Average area count of Propranolol peak in standard preparation.
WT	=	Weight of Propranolol sample in mg.
WS	=	Weight of Propranolol standard in mg.
D	=	Dilution factor
P	=	% purity of Propranolol standard (as is basis)
A	=	Average Wt of Propranolol tablets in mg
L	=	Label claim in mg

Validation

Following solutions were prepared for validation of method.

1. Identification

50.5 mg Propranolol standard was weighed accurately, in a 50 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase. Transfer 10 ml of the above solution to 100 ml and make up with mobile phase.

2. System suitability/System Precision

50.5 mg Propranolol standard was weighed accurately, in a 50 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase. Transfer 10 ml of the above solution to 100 ml and make up with mobile phase. This solution was injected six times, mean and RSD of detector response of Propranolol was calculated.

3. Specificity of the method

No interference from excipients

This was demonstrated by preparing a placebo containing all excipients and injecting a sample prepared from the same. There are no peaks at the retention time of Propranolol, thereby indicating that there is no interference from excipients.

Standard preparation

50.7 mg of Propranolol standard was weighed accurately and transferred to 50 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase. Transfer 10 ml of the above solution to 100 ml and make up with mobile phase.

Table 4.2.3a : Chromatographic conditions for Degradation study.

Degradation	Condition
Acid degradation	119.5 mg Propranolol tablet, and 2 ml 2M HCl. Heated at 70°C for 60 min.
Alkali degradation	114.5 mg Propranolol tablet,and 2ml 2M NaOH. Heated at 70°C for 60 min.
Peroxide degradation	117.9 mg Propranolol tablet, and 1 ml of 30.0% H_2O_2. Heated at 70°C for 60 min.
Sun-Light exposure	114.9 mg Propranolol tablet kept for 2 hrs. in

	Sun-light.
Thermal degradation	115.3 mg Propranolol tablet and 20ml mobile phase. Heated at 70°C for 60 min.

4. Linearity Study

The linearity of detector (UV) response for Propranolol standard was determined by preparing and injecting solutions in the concentration range of 50 – 150μg/ml (50-150 % of assay conc.) of Propranolol standard.

Stock Solution

250.3 mg of Propranolol standard was weighed accurately and transferred to a 50 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min. volume was made with mobile phase.

Table 4.2.3b : Solution preparation for linearity study of Propranolol tablets

% level of standard	Vol. of stock solution (ml)	Final dil (ml)	Final concentrations of Propranolol (μg/ml)
50	1.0	100	50.06
75	1.5	100	75.09
100	2.0	100	100.12
125	2.5	100	125.15
150	3.0	100	150.18

5. Method Precision

Standard preparation

50.5 mg of Propranolol standard was weighed accurately and transferred to 50 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase mixed thoroughly. Transfer 10 ml of the above solution to 100 ml and make up with mobile phase.

Sample preparation

Six sets of Propranolol tablets of same lot were prepared as per the method on same day for analysis by the HPLC method . Table 4.2.3c shows solution preparation for method precision sets.

Table 4.2.3c : Solution preparation for method precision sets of Propranolol Tablets

Set no.	Weight of sample(mg)	Final Dilution (ml)
1	110.5	100
2	110.1	100
3	109.2	100
4	110.9	100
5	110.0	100
6	111.0	100

Note: Average weight of 10 mg(content/tablet) of Propranolol was calculated to be 110.2 mg (average wt of 10 tablets were observed).

6. Recovery(Accuracy)

Recovery study was performed by spiking Propranolol standard in the placebo at levels 80%, 100% & 120 % of assay concentration in triplicate. The samples were chromatographed according to the assay procedure. Table 4.2.3d shows solution preparation for recovery study.

Table 4.2.3d : Solution preparation for recovery study of Propranolol Tablets

Solution detail	Placebo wt(mg)	Volume of stock solution (ml) (linearity study)	Final Dilution (ml)
standard	-	2.0	100
80 %	100.1	1.6	100
100%	99.8	2.0	100
120%	99.5	2.4	100

7. Ruggedness

Method ruggedness was established by analysing sample at normal operating condition and at changed conditions then results were compared. Table no.4.2.3e shows details of solution preparation for ruggedness study.

Table 4.2.3e : Solution preparation for ruggedness study of Propranolol Tablets

Parameters	Solution	Weight (mg)	Final dilution (ml)		
Original	Standard	50.5	50	10	100
	Sample	110.5	100	-	-
Changed	Standard	50.6	50	10	100
	Sample	110.6	100	-	-

8. Stability of analytical solution

System precision study solution was injected in duplicate at different time intervals and peak areas were recorded.

4.2.4 Terazosin

The following solutions are required to be prepared for routine analysis of calculation of assay of Terazosin tablet.

System suitability/Standard solution

Transfer about 50 mg of Terazosin standard, accurately weighed, to a 50 ml volumetric flask. Dissolve in and dilute up to mark with mobile phase. Transfer 1 ml of the above solution to 100 ml and make up with mobile phase.

Sample preparation

Transfer blend of Terazosin tablets equivalent to 1 mg of Terazosin to a 100 ml volumetric flask. Add about 20 ml of mobile phase and sonicate for 5 minutes with

intermediate shaking and dilute with mobile phase to volume and mix. Centrifuge this solution at about 2000 RPM for 10 min. and use upper clear solution for injection.

Calculations

$$\%\text{ Assay of Terazosin} = \frac{AT}{AS} \times \frac{WS}{WT} \times D \times A \times \frac{P}{L}$$

Where :

AT = Average area count of Terazosin peak in sample preparation.

AS = Average area count of Terazosin peak in standard preparation.

WT = Weight of Terazosin sample in mg.

WS = Weight of Terazosin standard in mg.

D = Dilution factor

P = % purity of Terazosin standard (as is basis)

A = Average Wt of Terazosin tablets in mg

L = Label claim in mg

Validation

Following solutions were prepared for validation of method.

1. Identification

50.6 mg Terazosin standard was weighed accurately, in a 50 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase. Transfer 1ml of the above solution to 100 ml and make up with mobile phase.

2. System suitability/System Precision

50.6 mg Terazosin standard was weighed accurately, in a 50 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase. Transfer 1 ml of the above solution to 100 ml and make up with mobile phase. This solution was injected six times, mean and RSD of detector response of Terazosin was calculated.

3. Specificity of the method

No interference from excipients

This was demonstrated by preparing a placebo containing all excipients and injecting a sample prepared from the same. There are no peaks at the retention time of Terazosin, thereby indicating that there is no interference from excipients.

Standard preparation

50.5 mg of Terazosin standard was weighed accurately and transferred to 50 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase. Transfer 1 ml of the above solution to 100 ml and make up with mobile phase.

Table 4.2.4a : Chromatographic conditions for Degradation study.

Degradation	Condition
Acid degradation	117.2mg Terazosin tablet, and 2 ml 2M HCl. Heated at 70°C for 60 min.
Alkali degradation	116.3 mg Terazosin tablet,and 2ml 2M NaOH. Heated at 70°C for 60 min.
Peroxide degradation	118.2 mg Terazosin tablet, and 1 ml of 30.0% H_2O_2. Heated at 70°C for 60 min.
Sun-Light exposure	125.9 mg Terazosin tablet kept for 2 hrs. in

	Sun-light.
Thermal degradation	120.2 mg Terazosin tablet and 20ml mobile phase. Heated at 70°C for 60 min.

4. Linearity Study

The linearity of detector (UV) response for Terazosin standard was determined by preparing and injecting solutions in the concentration range of 5 –15μg/ml (50-150 % of assay conc.) of Terazosin standard.

Stock Solution

51.1 mg of Terazosin standard was weighed accurately and transferred to a 100 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min. volume was made with mobile phase. This solution was diluted as shown in Table - 4.2.4b

Table 4.2.4b : Solution preparation for linearity study of Terazosin tablets

% level of standard	Vol. of stock solution (ml)	Final Dilution (ml)	Final concentrations of Terazosin (μg/ml)
50	1.0	100	5.11
75	1.5	100	7.67
100	2.0	100	10.22
125	2.5	100	12.78
150	3.0	100	15.33

5. Method Precision

Standard preparation

50.6 mg of Terazosin standard was weighed accurately and transferred to 50 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase mixed thoroughly. Transfer 1 ml of the above solution to 100 ml and make up with mobile phase.

Sample preparation

Six sets of Terazosin tablets of same lot were prepared as per the method on same day for analysis by the HPLC method . Table 4.2.4c shows solution preparation for method precision sets.

Table 4.2.4c : Solution preparation for method precision sets of Terazosin Tablets

Set no.	Weight of sample(mg)	Final Dilution (ml)
1	122.2	100
2	123.9	100
3	124.1	100
4	125.3	100
5	123.6	100
6	123.9	100

Note: Average weight of 1 mg(content/tablet) of Terazosin was calculated to be 117.2 mg (average wt of 10 tablets were observed).

6. Recovery(Accuracy)

Recovery study was performed by spiking Terazosin standard in the placebo at levels 80%, 100% & 120 % of assay concentration in triplicate. The samples were chromatographed according to the assay procedure. Table 4.2.4d shows solution preparation for recovery study.

Table 4.2.4d : Solution preparation for recovery study of Terazosin Tablets

Solution detail	Placebo wt(mg)	Volume of stock solution (ml) (linearity study)	Final Dilution (ml)
standard	-	2.0	100
80 %	116.2	1.6	100
100%	116.5	2.0	100
120%	116.9	2.4	100

7. Ruggedness

Method ruggedness was established by analysing sample at normal operating condition and at changed conditions then results were compared. Table no.4.2.4e shows details of solution preparation for ruggedness study.

Table 4.2.4e : Solution preparation for ruggedness study of Terazosin Tablets

Parameters	Solution	Weight (mg)	Final dilution (ml)		
Original	Standard	50.6	50	1	100
	Sample	122.2	100	-	-
Changed	Standard	50.1	50	1	100
	Sample	120.8	100	-	-

8. Stability of analytical solution

System precision study solution was injected in duplicate at different time intervals and peak areas were recorded.

4.2.5 Verapamil

The following solutions are required to be prepared for routine analysis of calculation of assay of Verapamil tablet.

System suitability/Standard solution

Transfer about 50 mg of Verapamil standard, accurately weighed, to a 25 ml volumetric flask. Dissolve in and dilute up to mark with mobile phase. Transfer 10 ml of the above solution to 100 ml and make up with mobile phase.

Sample preparation

Transfer blend of Verapamil tablets equivalent to 20 mg of Verapamil to a 100 ml volumetric flask. Add about 20 ml of mobile phase and sonicate for 5 minutes with

intermediate shaking and dilute with mobile phase to volume and mix. Centrifuge this solution at about 2000 RPM for 10 min. and use upper clear solution for injection.

Calculations

$$\%\ \text{Assay of Verapamil} = \frac{AT}{AS} \times \frac{WS}{WT} \times D \times A \times \frac{P}{L}$$

Where :

AT = Average area count of Verapamil peak in sample preparation.

AS = Average area count of Verapamil peak in standard preparation.

WT = Weight of Verapamil sample in mg.

WS = Weight of Verapamil standard in mg.

D = Dilution factor

P = % purity of Verapamil standard (as is basis)

A = Average Wt of Verapamil tablets in mg

L = Label claim in mg

Validation

Following solutions were prepared for validation of method.

1. Identification

51.9 mg Verapamil standard was weighed accurately, in a 25 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase. Transfer 10 ml of the above solution to 100 ml and make up with mobile phase.

2. System suitability/System Precision

51.9 mg Verapamil standard was weighed accurately, in a 25 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase. Transfer 10 ml of the above solution to 100 ml and make up with mobile phase. This solution was injected six times, mean and RSD of detector response of Verapamil was calculated.

3. Specificity of the method

No interference from excipients
This was demonstrated by preparing a placebo containing all excipients and injecting a sample prepared from the same. There are no peaks at the retention time of Verapamil, thereby indicating that there is no interference from excipients.

Standard preparation
52.25 mg of Verapamil standard was weighed accurately and transferred to 25 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase. Transfer 10 ml of the above solution to 100 ml and make up with mobile phase.

Table 4.2.5a : Chromatographic conditions for Degradation study.

Degradation	Condition
Acid degradation	40.0mg Verapamil tablet, and 2 ml 2M HCl. Heated at 70°C for 60 min.
Alkali degradation	40.1 mg Verapamil tablet,and 2ml 2M NaOH. Heated at 70°C for 60 min.
Peroxide degradation	38.6 mg Verapamil tablet, and 1 ml of 30.0% H_2O_2. Heated at 70°C for 60 min.
Sun-Light exposure	42.6 mg Verapamil tablet kept for 2 hrs. in Sun-light.
Thermal degradation	40.2 mg Verapamil tablet and 20ml mobile phase. Heated at 70°C for 60 min.

4. Linearity Study

The linearity of detector (UV) response for Verapamil standard was determined by preparing and injecting solutions in the concentration range of 100 –300μg/ml (50-150 % of assay conc.) of Verapamil standard.

Stock Solution

251.2 mg of Verapamil standard was weighed accurately and transferred to a 25 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min. volume was made with mobile phase. This solution was diluted as shown in Table - 4.2.5b

Table 4.2.5b : Solution preparation for linearity study of Verapamil tablets

% level of standard	Vol. of stock solution (ml)	Final Dilution (ml)	Final concentrations of Verapamil (μg/ml)
50	1.0	100	100.48
75	1.5	100	150.72
100	2.0	100	200.96
125	2.5	100	251.20
150	3.0	100	301.44

5. Method Precision

Standard preparation

51.9 mg of Verapamil standard was weighed accurately and transferred to 25 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase mixed thoroughly. Transfer 10 ml of the above solution to 100 ml and make up with mobile phase.

Sample preparation

Six sets of Verapamil tablets of same lot were prepared as per the method on same day for analysis by the HPLC method . Table 4.2.5c shows solution preparation for method precision sets.

Table 4.2.5c : Solution preparation for method precision sets of Verapamil Tablets

Set no.	Weight of sample(mg)	Final Dilution (ml)
1	41.5	100
2	41.4	100
3	42.2	100
4	42.0	100
5	42.2	100
6	42.0	100

Note: Average weight of 40 mg(content/tablet) of Verapamil was calculated to be 83.1 mg (average wt of 10 tablets were observed).

6. Recovery(Accuracy)

Recovery study was performed by spiking Verapamil standard in the placebo at levels 80%, 100% & 120 % of assay concentration in triplicate. The samples were chromatographed according to the assay procedure. Table 4.2.5d shows solution preparation for recovery study.

Table 4.2.5d : Solution preparation for recovery study of Verapamil Tablets

Solution detail	Placebo wt(mg)	Volume of stock solution (ml) (linearity study)	Final Dilution (ml)
standard	-	2.0	100
80 %	41.6	1.6	100
100%	41.3	2.0	100
120%	41.5	2.4	100

7. Ruggedness

Method ruggedness was established by analysing sample at normal operating condition and at changed conditions then results were compared. Table no.4.2.5e shows details of solution preparation for ruggedness study.

Table 4.2.5e : Solution preparation for ruggedness study of Verapamil Tablets

Parameters	Solution	Weight (mg)	Final dilution (ml)		
Original	Standard	51.9	25	10	100
	Sample	41.5	100	-	-
Changed	Standard	50.7	25	10	100
	Sample	40.9	100	-	-

8. Stability of analytical solution

System precision study solution was injected in duplicate at different time intervals and peak areas were recorded.

4.2.6 Citalopram

The following solutions are required to be prepared for routine analysis of calculation of impurities of Citalopram bulk drug.

Standard solution

Solution A
Transfer about 5 mg each of impurities, accurately weighed, to a 200 ml volumetric flask. Dissolve in and dilute up to mark with mobile phase.

Solution B
Transfer about 2.5 mg each of Citalopram standard, accurately weighed, to a 200 ml volumetric flask. Dissolve in and dilute up to mark with mobile phase.

Solution C

Transfer about 2ml of solution A and 2 ml of solution B into a 25 ml volumetric flask. Dissolve in and dilute up to mark with mobile phase.

Sample preparation

Transfer 25 mg of Citalopram sample to a 25 ml volumetric flask. Dissolve and dilute with mobile phase upto the mark.

Calculations

$$\%\ \text{impurities(known) of Citalopram} = \frac{R_{sample}}{R_{impurity}} \times \frac{C_{impurity}}{C_{sample}} \times P$$

Where :

R_{sample} = Detector response of individual impurities in sample preparation.

$R_{impurity}$ = Detector response of individual impurities in standard preparation.

$C_{impurity}$ = Concentration of individual impurities in standard preparation(μg/ml)

C_{sample} = Concentration of sample preparation(μg/ml)

P = % purity of individual impurities.

$$\%\ \text{impurities(unknown) of Citalopram} = \frac{R_{sample}}{R_{impurity}} \times \frac{C_{impurity}}{C_{sample}} \times P$$

Where :

R_{sample} = Detector response of unknown impurities in sample preparation.

$R_{impurity}$ = Detector response of Citalopram in standard preparation.

$C_{impurity}$ = Concentration of Citalopram in standard preparation(μg/ml)

C_{sample} = Concentration of sample preparation(μg/ml)

P = % purity of citalopram.

Limit of impurities(As per ICH Guidelines)

1) Any single known impurities not more than 0.2%.

2) Any single unknown impurity not more than 0.1 %

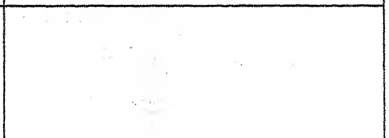
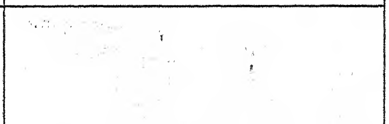
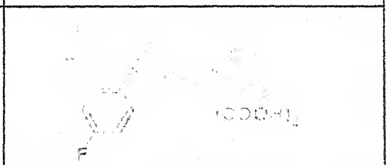
Validation

Following solutions were prepared for validation of method.

1. Identification

About 1 mg of individual impurites and citalopram standard separately was weighed accurately, in a 10 ml volumetric flask containing 5 ml of mobile phase each. After sonication for 10 min, volume was made with mobile phase.

Table 4.2.6a : Chemical names and structure of impurities of Citalopram bulk drug

Impurities	Chemical Name	Chemical Structure
Impurity 1	1-(3-Dimethylaminopropyl)-1-(4-fluorophenyl)-1,3dihydro-isobenzofurn-5-carboxylic acid amide.	
Impurity 2	1-(3-Dimethylaminopropyl,N oxide)-1-(4-fluorophenyl)-5-phthalan carbonitrile.	
Impurity 3	1-(3-Methylaminopropyl)-1-(4-fluorophenyl)-5-cyanophthalan oxalate.	
Impurity 4	1-(3-dimethylaminopropyl)-1-(4-fluorophenyl)-phthalan oxalate.	
Impurity 5	1-(3-dimethylaminopropyl)-1-(phenyl)-5-phthalan carbonitrile oxalate.	

Impurity 6	1-(3-dimethylaminopropyl)-1-(4-fluorophenyl)-5-chlorophthalan oxalate	
Impurity 7	1-(3-dimethylaminopropyl)-1-(4-fluorophenyl)-5-bromophthalan oxalate.	

2. System suitability

1.1 mg Citalopram standard and 1.0 mg of impurity 5 was weighed accurately, in a 10 ml volumetric flask containing 5 ml of mobile phase. After sonication for 2 min, volume was made with mobile phase. 20µl of the solution was injected into chromatographic system and the resolution factor was calculated.

3. Instrument Precision

Solution A
Transfer about 5.7 mg, 6.1 mg, 6.0 mg, 5.3 mg, 6.1 mg, 6.1 mg and 5.6 mg each of impurities 1,2,3,4,5,6 and 7 accurately weighed, to a 200 ml volumetric flask. Containing 10 ml of mobile phase. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution B
Transfer about 2.52 mg each of Citalopram standard, accurately weighed, to a 200 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution C
Transfer about 2ml of solution A and 2 ml of solution B into a 25 ml volumetric flask. Dissolve in and dilute up to mark with mobile phase.

Solution C was injected six times and mean ,% RSD of individual impurities and Citalopram were observed.

4. Specificity of the method

Standard preparation

25.2 mg of Citalopram standard was weighed accurately and transferred to 25 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase.

Table 4.2.6b : Chromatographic conditions for Degradation study.

Degradation	Condition
Acid degradation	25.1mg Citalopram bulk drug, and 5 ml 2M HCl. Heated at 100°C for 10 min.
Alkali degradation	25.1mg Citalopram bulk drug,and 5ml 2M NaOH. Heated at 100°C for 10 min.
Peroxide degradation	24.9mg Citalopram bulk drug, and 5 ml of 30.0% H_2O_2. Heated at 100°C for 10 min.
Sun-Light exposure	25.1mg Citalopram bulk drug kept for 4 hrs. in Sun-light.
Thermal degradation	24.9mg Citalopram bulk drug and 20ml mobile phase. Heated at 100°C for 10 min.

Acid and Alkali degradation pH was adjusted to 7.0 before injection into the system.

5. Linearity Study

The linearity of detector (UV) response for impurities was determined by preparing and injecting solutions in the concentration range of 1.0 –3.0μg/ml (50-150 % of limit conc.) of Citalopram standard.

Stock Solution

Solution A

Transfer about 5.7 mg, 6.1 mg, 6.0 mg, 5.3 mg, 6.1 mg, 6.1 mg and 5.6 mg each of impurities 1,2,3,4,5,6 and 7 accurately weighed, to a 200 ml volumetric flask

Containing 10 ml of mobile phase. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution B
Transfer about 2.52 mg each of Citalopram standard, accurately weighed, to a 200 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase. This solution was diluted as shown in Table - 4.2.6c

Table 4.2.6c : Solution preparation for linearity study of Citalopram bulk drugs

% level of std	Vol. of stock solution A & B each (ml)	Final Dil (ml)	Final concentrations (μg/ml)							
			Imp 1	Imp 2	Imp 3	Imp 4	Imp 5	Imp 6	Imp 7	Citalo pram
50	1.0	25	1.14	1.22	1.20	1.06	1.22	1.22	1.12	0.50
75	1.5	25	1.71	1.83	1.80	1.59	1.83	1.83	1.68	0.76
100	2.0	25	2.28	2.44	2.40	2.12	2.44	2.44	2.24	1.01
125	2.5	25	2.85	3.05	3.00	2.65	3.05	3.05	2.80	1.26
150	3.0	25	3.42	3.66	3.60	3.18	3.66	3.66	3.36	1.51

6. Method Precision

Stock Solution

Solution A
Transfer about 5.7 mg, 6.1 mg, 6.0 mg, 5.3 mg, 6.1 mg, 6.1 mg and 5.6 mg each of impurities 1,2,3,4,5,6 and 7 accurately weighed, to a 200 ml volumetric flask Containing 10 ml of mobile phase. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution B
Transfer about 2.52 mg each of Citalopram standard, accurately weighed, to a 200 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution C
2 ml of solution A and 2 ml of solution B were transferred into 25 ml volumetric flask.

Mixed and diluted to volume with mobile phase.

Standard preparation
Six consecutive injections of solution C were done.

Sample preparation

Six sets of Citalopram were prepared as per the method on same day for analysis by the HPLC method.Table 4.2.6d shows solution preparation for method precision sets.

Table 4.2.6d : Solution preparation for method precision sets of Citalopram Bulk drug

Wt. of std (mg)	Vol. of stock solution A (ml)	Final Dil. (ml)	Final concentrations (μg/ml)							
			Imp 1	Imp 2	Imp 3	Imp 4	Imp 5	Imp 6	Imp 7	Citalo pram
25.2	2.0	25	2.28	2.44	2.40	2.12	2.44	2.44	2.24	1008.0
25.1	2.0	25	2.28	2.44	2.40	2.12	2.44	2.44	2.24	1004.0
25.1	2.0	25	2.28	2.44	2.40	2.12	2.44	2.44	2.24	1004.0
25.3	2.0	25	2.28	2.44	2.40	2.12	2.44	2.44	2.24	1012.0
25.1	2.0	25	2.28	2.44	2.40	2.12	2.44	2.44	2.24	1004.0
25.2	2.0	25	2.28	2.44	2.40	2.12	2.44	2.44	2.24	1008.0

7. Recovery(Accuracy)

Recovery study was performed by spiking impurities in the citalopram standard at levels 70%, 85%,100%,115% & 130 % of limit concentration(0.2%). The samples were chromatographed in triplicate according to the procedure. Table 4.2.6e shows solution preparation for recovery study.

Stock Solution

Solution A
Transfer about 5.7 mg, 6.1 mg, 6.0 mg, 5.3 mg, 6.1 mg, 6.1 mg and 5.6 mg each of impurities 1,2,3,4,5,6 and 7 accurately weighed, to a 200 ml volumetric flask

Containing 10 ml of mobile phase. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution B

Transfer about 2.52 mg each of Citalopram standard, accurately weighed, to a 200 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution C

2 ml of solution A and 2 ml of solution B were transferred into 25 ml volumetric flask. Mixed and diluted to volume with mobile phase.

Standard preparation

Six consecutive injections were done of solution C.

Table 4.2.6e : Solution preparation for recovery study of Citalopram

% level std	wt std (mg)	Vol of stock sol A (ml)	Final Dil. (ml)	Final Concentrations (μg/ml)							
				Imp 1	Imp 2	Imp 3	Imp 4	Imp 5	Imp 6	Imp 7	Std
70	25.2	1.4	25	1.60	1.71	1.68	1.48	1.71	1.71	1.57	1008
85	25.1	1.7	25	1.94	2.07	2.04	1.80	2.07	2.07	1.90	1004
100	25.0	2.0	25	2.28	2.44	2.40	2.12	2.44	2.44	2.24	1000
115	25.2	2.3	25	2.62	2.81	2.76	2.44	2.81	2.81	2.58	1008
130	25.3	2.6	25	2.96	3.17	3.12	2.76	3.17	3.17	2.91	1012

8. Limit of detection and Limit of quantitation

For determining the limit of detection (LOD) and limit of quantitation (LOQ), the method based on the residual standard deviation of a regression line and slope was adopted.

Stock Solution

Solution A
Transfer about 5.7 mg, 6.1 mg, 6.0 mg, 5.3 mg, 6.1 mg, 6.1 mg and 5.6 mg each of impurities 1,2,3,4,5,6 and 7 accurately weighed, to a 200 ml volumetric flask Containing 10 ml of mobile phase. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution B
Transfer about 2.52 mg each of Citalopram standard, accurately weighed, to a 200 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution C
1 ml of solution A and1 ml of solution B were transferred into 25 ml volumetric flask. Mixed and diluted to volume with mobile phase.

Table 4.2.6 f : Solution preparation for Limit of detection and quantitation study of Citalopram

Sol No.	Vol. of stock sol C (ml)	Final Dil (ml)	Final concentrations (µg/ml)							
			Imp1	Imp2	Imp3	Imp4	Imp5	Imp6	Imp7	Citalo pram
1	1	1	1.140	1.220	1.200	1.060	1.220	1.220	1.120	0.500
2	5	10	0.570	0.610	0.600	0.530	0.610	0.610	0.560	0.250
3	2.5	10	0.285	0.305	0.300	0.265	0.305	0.305	0.280	0.125
4	1.2	10	0.137	0.146	0.144	0.127	0.146	0.146	0.134	0.060
5	0.6	10	0.068	0.073	0.072	0.064	0.073	0.073	0.067	0.030
6	0.3	10	0.034	0.037	0.036	0.032	0.037	0.037	0.034	0.015

9. Ruggedness

Method ruggedness was established by analysing sample at normal operating condition and at changed conditions then results were compared. Table no.4.2.6g shows details of solution preparation for ruggedness study.

Stock Solution

Solution A

Transfer about 5.7 mg, 6.1 mg, 6.0 mg, 5.3 mg, 6.1 mg, 6.1 mg and 5.6 mg each of impurities 1,2,3,4,5,6 and 7 accurately weighed, to a 200 ml volumetric flask Containing 10 ml of mobile phase. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution B

Transfer about 2.52 mg each of Citalopram standard, accurately weighed, to a 200 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Table 4.2.6g: Solution preparation for ruggedness study of Citalopram Tablets

Parameter Changed	Condition	Solution	Wt (mg)	Vol. of stock A (ml)	Vol. of stock B (ml)	Final Dilution (ml)
Original	As described in method	Standard	-	2	2	25
		Sample	25.2	2	-	25
Analyst	Analysis performed by another analyst, other conditions constant	Standard	-	2	2	25
		Sample	25.3	2	-	25
Column	Phenomenex Luna(C18, 250 *4.6 mm,5 µ) other conditions constant	Standard	-	2	2	25
		Sample	25.1	2	-	25
Column	Column temp. was changed t	Standard	-	2	2	25

Temp.	30°C. other conditions constant	Sample	25.4	2	-	25
Mobile phase Composition	Buffer:methanol:acetonitrile::50:42.5:7.5. other conditions constant	Standard	-	2	2	25
		Sample	25.2	2	-	25
Instrument change	Instrument configuration was changed. other conditions constant	Standard	-	2	2	25
		Sample	25.1	2	-	25

10. Stability of analytical solution

System precision study solution was injected in duplicate at different time intervals and peak areas were recorded.

4.2.7 Metaxalone

The following solutions are required to be prepared for routine analysis of calculation of impurities of Metaxalone bulk drug.

Standard solution

Solution A

Transfer about 25.0 mg each of impurities, accurately weighed, to a 100 ml volumetric flask. Dissolve in and dilute up to mark with mobile phase.

Solution B

Transfer about 25.0 mg each of Metaxalone standard, accurately weighed, to a 100 ml volumetric flask. Dissolve in and dilute up to mark with mobile phase.

Solution C

Transfer about 10 ml of solution A and 5 ml of solution B into a 100 ml volumetric flask. Dissolve in and dilute up to mark with mobile phase.

Solution D

Transfer about 2 ml of solution C into a 25 ml volumetric flask. Dissolve in and dilute up to mark with mobile phase.

Sample preparation

Transfer 25 mg of Metaxalone sample to a 25 ml volumetric flask. Dissolve and dilute with mobile phase upto the mark.

Calculations

$$\text{\% impurities(known) of Metaxalone} = \frac{R_{sample}}{R_{impurity}} \times \frac{C_{impurity}}{C_{sample}} \times P$$

Where :

R_{sample} = Detector response of individual impurities in sample preparation.

$R_{impurity}$ = Detector response of individual impurities in standard preparation.

$C_{impurity}$ = Concentration of individual impurities in standard preparation(µg/ml)

C_{sample} = Concentration of sample preparation(µg/ml)

P = % purity of individual impurities.

$$\text{\% impurities(unknown) of Metaxalone} = \frac{R_{sample}}{R_{impurity}} \times \frac{C_{impurity}}{C_{sample}} \times P$$

Where :

R_{sample} = Detector response of unknown impurities in sample preparation.

$R_{impurity}$ = Detector response of Metaxalone in standard preparation.

$C_{impurity}$ = Concentration of Metaxalone in standard preparation(µg/ml)

C_{sample} = Concentration of sample preparation(µg/ml)

P = % purity of Metaxalone.

Limit of impurities(As per ICH Guidelines)

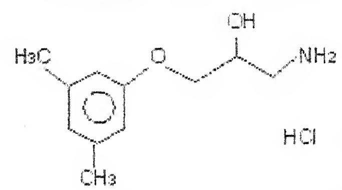
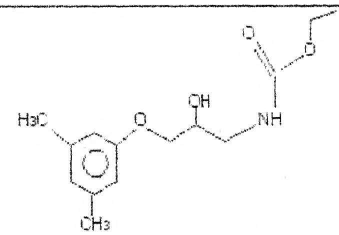
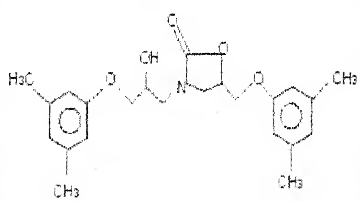
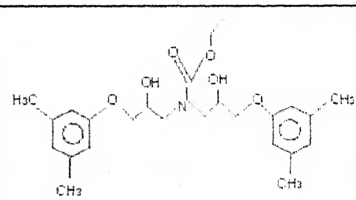
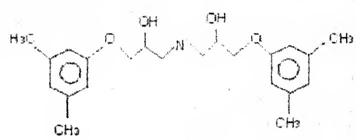
1) Any single known impurities not more than 0.2%.

2) Any single unknown impurity not more than 0.1 %

Validation
Following solutions were prepared for validation of method.
1. Identification

About 1 mg of individual impurites and Metaxalone standard was weighed accurately, in a 10 ml volumetric flask containing 5 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase.

Table 4.2.7a : Chemical names and structure of impurities of Metaxalone bulk drug

Impurities	Chemical Name	Chemical Structure
Impurity 1	3-(3,5-Dimethylphenoxy)-2-hydroxypropylamine hydrochloride	
Impurity 2	Ethyl [3-(3,5-dimethylphenoxy)-2-hydroxypropyl] carbamate	
Impurity 3	3-[3-(3,5-dimethylphenoxy)-2-hydroxypropyl]-5-(3,5-methylphenoxy)methyl-oxazolidine-2-one	
Impurity 4	Ethyl N,N-bis[3-(3,5-dimethylphenoxy)-2-hydroxypropyl] carbamate	
Impurity 5	N, N-bis[3-(3,5-dimethylphenoxy)-2-hydroxypropyl] amine	

2. System suitability

2.0 mg Metaxalone standard and 2.0 mg of impurity 2 was weighed accurately, in a 10 ml volumetric flask containing 5 ml of mobile phase. After sonication for 2 min, volume was made with mobile phase. 20µl of the solution was injected into chromatographic system and the resolution factor was calculated.

3. Instrument Precision

Solution A

Transfer about 24.3 mg, 25.2 mg, 26.0 mg, 25.3 mg and 25.9 mg, each of impurities 1,2,3,4 and 5 accurately weighed, to a 100 ml volumetric flask Containing 10 ml of mobile phase. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution B

Transfer about 24.8 mg each of Metaxalone standard, accurately weighed, to a 100 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution C

10 ml of solution A and 5 ml of solution B is diluted to 100 ml by mobile phase.

Solution D

Transfer about 2 ml of solution C into a 25 ml volumetric flask. Dissolve in and dilute up to mark with mobile phase.

Solution D was injected six times and mean, % RSD of individual impurities and Metaxalone were observed.

4. Specificity of the method

Standard preparation
25.0 mg of Metaxalone standard was weighed accurately and transferred to 25 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase.

Table 4.2.7b : Chromatographic conditions for Degradation study.

Degradation	Condition
Acid degradation	25.1mg Metaxalone bulk drug, and 5 ml 5.0M HCl. Heated at 100°C for 10 min.
Alkali degradation	25.1mg Metaxalone bulk drug,and 5ml 2M NaOH. Heated at 100°C for 10 min.
Peroxide degradation	25.3mg Metaxalone bulk drug, and 5 ml of 30.0% H_2O_2. Heated at 100°C for 10 min.
Sun-Light exposure	25.1mg Metaxalone bulk drug kept for 4 hrs. in Sun-light.
Thermal degradation	24.9mg Metaxalone bulk drug and 20ml mobile phase. Heated at 100°C for 10 min.

Acid and Alkali degradation pH was adjusted to 7.0 before injection into the system.

5. Linearity Study

The linearity of detector (UV) response for impurities was determined by preparing and injecting solutions in the concentration range of 1.0 –3.0μg/ml (50-150 % of limit conc.) of Metaxalone standard.

Stock Solution

Solution A
Transfer about 24.3 mg, 25.2 mg, 26.0 mg, 25.3 mg and 25.9 mg, each of impurities 1,2,3,4 and 5 accurately weighed, to a 100 ml volumetric flask Containing 10 ml of mobile phase. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution B
Transfer about 24.8 mg each of Metaxalone standard, accurately weighed, to a 100 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution C
10 ml of solution A and 5 ml of solution B is diluted to 100 ml by mobile phase. This solution was diluted as shown in Table - 4.2.7c

Table 4.2.7c : Solution preparation for linearity study of Metaxalone bulk drugs

% level of std	Vol. of stock solution C (ml)	Final Dil (ml)	Final concentrations (µg/ml)					
			Imp 1	Imp 2	Imp 3	Imp 4	Imp 5	Metaxalone
50	1.0	25	0.97	1.01	1.04	1.01	1.04	0.50
75	1.5	25	1.46	1.51	1.56	1.52	1.55	0.74
100	2.0	25	1.94	2.02	2.08	2.02	2.07	0.99
125	2.5	25	2.43	2.52	2.60	2.53	2.59	1.24
150	3.0	25	2.92	3.02	3.12	3.04	3.11	1.49

6. Method Precision

Stock Solution

Solution A
Transfer about 24.5 mg, 24.8 mg, 25.1 mg, 25.0 mg and 25.4 mg, each of impurities 1,2,3,4 and 5 accurately weighed, to a 100 ml volumetric flask Containing 10 ml of mobile phase. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution B

Transfer about 25.0 mg each of Metaxalone standard, accurately weighed, to a 100 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution C

10 ml of solution A and 5 ml of solution B is diluted to 100 ml by mobile phase.

Solution D

10 ml of solution A is diluted to 100 ml by mobile phase.

Standard preparation

2 ml of solution C was diluted to 25 ml by mobile phase and six consecutive injections were done.

Sample preparation

Six sets of Metaxalone were prepared as per the method on same day for analysis by the HPLC method . Table 4.2.7e shows solution preparation for method precision sets.

Table 4.2.7d : Solution preparation for method precision sets of Metaxalone Bulk drug

Wt. of std (mg)	Vol. of stock solution D (ml)	Final Dil. (ml)	Final concentrations (µg/ml)					
			Imp 1	Imp 2	Imp 3	Imp 4	Imp 5	Metaxalone
24.8	2.0	25	1.96	1.98	2.01	2.00	2.03	992
24.9	2.0	25	1.96	1.98	2.01	2.00	2.03	996
24.8	2.0	25	1.96	1.98	2.01	2.00	2.03	992
25.1	2.0	25	1.96	1.98	2.01	2.00	2.03	1004
24.7	2.0	25	1.96	1.98	2.01	2.00	2.03	988
25.0	2.0	25	1.96	1.98	2.01	2.00	2.03	1000

7. Recovery(Accuracy)

Recovery study was performed by spiking impurities in the Metaxalone standard at levels 70%, 85%,100%,115% & 130 % of limit concentration(0.2%). The samples were chromatographed in triplicate according to the procedure. Table 4.2.7e shows solution preparation for recovery study.

Stock Solution

Solution A
Transfer about 24.5 mg, 24.8 mg, 25.1 mg, 25.0 mg and 25.4 mg, each of impurities 1,2,3,4 and 5 accurately weighed, to a 100 ml volumetric flask Containing 10 ml of mobile phase. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution B
Transfer about 25.0 mg each of Metaxalone standard, accurately weighed, to a 100 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution C
10 ml of solution A and 5 ml of solution B is diluted to 100 ml by mobile phase.

Solution D
10 ml of solution A is diluted to 100 ml by mobile phase.

Standard preparation
2 ml of solution C was diluted to 25 ml by mobile phase and six consecutive injections were done.

Table 4.2.7e : Solution preparation for recovery study of Metaxalone

% level std	wt std (mg)	Vol of stock sol D (ml)	Final Dil. (ml)	Final Concentrations (μg/ml)					
				Imp 1	Imp 2	Imp 3	Imp 4	Imp 5	Metaxalone
70	25.3	1.4	25	1.36	1.39	1.43	1.42	1.46	1012
85	25.2	1.7	25	1.65	1.69	1.74	1.72	1.77	1008
100	25.0	2.0	25	1.94	1.99	2.05	2.02	2.08	1000
115	25.3	2.3	25	2.24	2.29	2.36	2.33	2.39	1008
130	25.0	2.6	25	2.53	2.59	2.66	2.63	2.70	1012

8. Limit of detection and Limit of quantitation

For determining the limit of detection (LOD) and limit of quantitation (LOQ), the method based on the residual standard deviation of a regression line and slope was adopted.

Stock Solution

Solution A

Transfer about 24.0 mg, 24.6 mg, 25.2 mg, 24.4 mg and 25.4 mg, each of impurities 1,2,3,4 and 5 accurately weighed, to a 100 ml volumetric flask Containing 10 ml of mobile phase. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution B

Transfer about 24.5 mg each of Metaxalone standard, accurately weighed, to a 100 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution C
10 ml of solution A and 5 ml of solution B is diluted to 100 ml by mobile phase.

Solution D
1 ml of solution C is diluted to 25 ml by mobile phase.

Table 4.2.7f : Solution preparation for Limit of detection and quantitation study of Metaxalone

Sol No.	Vol. of stock sol D (ml)	Final Dil (ml)	Final concentrations (μg/ml)					
			Imp1	Imp2	Imp3	Imp4	Imp5	Metaxalone
1	1	1	0.960	0.984	1.008	0.976	1.016	0.490
2	5	10	0.480	0.492	0.504	0.488	0.508	0.245
3	2.5	10	0.240	0.246	0.252	0.244	0.254	0.123
4	1.2	10	0.115	0.118	0.121	0.117	0.122	0.059
5	0.6	10	0.058	0.059	0.060	0.059	0.061	0.029
6	0.3	10	0.029	0.030	0.030	0.029	0.030	0.015
7	0.1	10	0.005	0.005	0.005	0.005	0.005	0.002

9. Ruggedness

Method ruggedness was established by analysing sample at normal operating condition and at changed conditions then results were compared. Table no.4.2.7g shows details of solution preparation for ruggedness study.

Stock Solution

Solution A
Transfer about 24.0 mg, 24.6 mg, 25.2 mg, 24.4 mg and 25.4 mg, each of impurities 1,2,3,4 and 5 accurately weighed, to a 100 ml volumetric flask Containing 10 ml of

mobile phase. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution B
Transfer about 24.5 mg each of Metaxalone standard, accurately weighed, to a 100 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution C
10 ml of solution A and 5 ml of solution B is diluted to 100 ml by mobile phase.

Solution D
10 ml of solution A is diluted to 100 ml by mobile phase.

Table 4.2.7g : Solution preparation for ruggedness study of Metaxalone Bulk drug

Parameter Changed	Condition	Solution	Wt (mg)	Vol. of stock C (ml)	Vol. of stock D (ml)	Final Dilution (ml)
Original	As described in method	Standard	-	2	-	25
		Sample	25.2	-	2	25
Analyst	Analysis performed by another analyst, other conditions constant	Standard	-	2	-	25
		Sample	25.3	-	2	25
Column	Phenomenex Luna(C8, 250 *4.6 mm,5 μ) other conditions constant	Standard	-	2	-	25
		Sample	25.1	-	2	25
Column Temp.	Column temp. was changed to 40°C. other conditions constant	Standard	-	2	-	25
		Sample	25.4	-	2	25
Mobile phase Composition	Buffer:acetonitrile::55:45,other conditions constant	Standard	-	2	-	25
		Sample	25.2	-	2	25
Instrument change	Instrument configuration was changed. other conditions constant	Standard	-	2	-	25
		Sample	25.1	-	2	25

10. Stability of analytical solution

Stock Solution

Solution A
Transfer about 24.3 mg, 25.2 mg, 26.0 mg, 25.3 mg and 25.9 mg, each of impurities 1,2,3,4 and 5 accurately weighed, to a 100 ml volumetric flask Containing 10 ml of mobile phase. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution B
Transfer about 24.8 mg each of Metaxalone standard, accurately weighed, to a 100 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution C
10 ml of solution A and 5 ml of solution B is diluted to 100 ml by mobile phase. Solution C was injected in duplicate at different time intervals and peak areas were recorded.

4.2.8 Ondansetron

The following solutions are required to be prepared for routine analysis of calculation of impurities of Ondansetron bulk drug.

Standard solution

Solution A
Transfer about 12.5 mg each of impurity A, impurity C and ondansetron standard accurately weighed, to a 25 ml volumetric flask. Dissolve in and dilute up to mark with mobile phase.

Solution B
Transfer about 12.5 mg each of Impurity D, accurately weighed, to a 50 ml volumetric flask. Dissolve in and dilute up to mark with mobile phase.

Solution C
Transfer about 5 ml of solution A and 5 ml of solution B into a 100 ml volumetric flask. Dissolve in and dilute up to mark with mobile phase.

Solution D
Transfer about 2 ml of solution C into a 50 ml volumetric flask. Dissolve in and dilute up to mark with mobile phase.

Sample preparation

Transfer 25 mg of Ondansetron sample to a 50 ml volumetric flask. Dissolve and dilute with mobile phase upto the mark.

Calculations

$$\text{\% impurities(known) of Ondansetron} = \frac{R_{sample}}{R_{impurity}} \times \frac{C_{impurity}}{C_{sample}} \times P$$

Where :

R_{sample} = Detector response of individual impurities in sample preparation.

$R_{impurity}$ = Detector response of individual impurities in standard preparation.

$C_{impurity}$ = Concentration of individual impurities in standard preparation(μg/ml)

C_{sample} = Concentration of sample preparation(μg/ml)

P = % purity of individual impurities.

$$\text{\% impurities(unknown) of Ondansetron} = \frac{R_{sample}}{R_{impurity}} \times \frac{C_{impurity}}{C_{sample}} \times P$$

Where :

R_{sample} = Detector response of unknown impurities in sample preparation.

$R_{impurity}$ = Detector response of Ondansetron in standard preparation.

$C_{impurity}$ = Concentration of Ondansetron in standard preparation(µg/ml)

C_{sample} = Concentration of sample preparation(µg/ml)

P = % purity of Ondansetron.

Limit of impurities(As per ICH Guidelines)

1) Any single known impurities(A and C) not more than 0.2% and impurity D not more than 0.1%(as per USP28–NF23 Page 1418)

2) Any single unknown impurity not more than 0.1 %

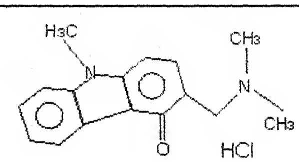
Validation

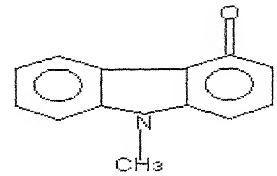
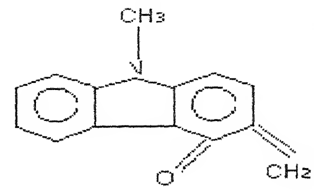
Following solutions were prepared for validation of method.

1. Identification

About 1.0 mg of each individual impurites and Ondansetron standard was weighed accurately, in a 10 ml volumetric flask containing 5 ml of mobile phase each. After sonication for 10 min, volume was made with mobile phase.

Table 4.2.8a : Chemical names and structure of impurities of Ondansetron bulk drug

Impurities	Chemical Name	Chemical Structure
Impurity A	3[(dimethylamino)methyl}-1,2,3,9-tetrahydro-9-methyl-4H-carbazol-4-one	

Impurity C	1,2,3,9-Tetrahydro-9-methyl-4H-carbazol-4-one	
Impurity D	1,2,3,9-Tetrahydro-9-methyl-3methylene-4H-carbazol-4-one	

Note: Nomenclature of impurites as A , C and D as per USP28–NF23 Page 1418

2. System suitability

5.0 mg impurity A and 10.0 mg of ondansetron standard was weighed accurately, in a 100 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase. 20µl of the solution was injected into chromatographic system and the resolution factor was calculated.

3. Instrument Precision

Solution A
Transfer 12.51 mg, 12.43 mg, each of impurities A and C accurately weighed, to a 25 ml volumetric flask Containing 10 ml of mobile phase. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution B
Transfer 12.55 mg of impurity D, accurately weighed, to a 50 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution C
Transfer 12.39 mg of ondansetron, accurately weighed, to a 25 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution D
5 ml of solution A ,B and C was pipetted out into a 100 ml volumetric flask and diluted by mobile phase upto the mark.

Instrument precision solution

Transfer about 2 ml of solution D into a 50 ml volumetric flask. Dissolve in and dilute up to mark with mobile phase.

Above mentioned solution was injected six times and mean, % RSD of individual impurities and Ondansetron were observed.

4. Specificity of the method
Standard preparation
25.6 mg of Ondansetron standard was weighed accurately and transferred to 50 ml volumetric flask containing 10 ml of mobile phase. After sonication for 10 min, volume was made with mobile phase.

Table 4.2.8b : Chromatographic conditions for Degradation study

Degradation	Condition
Acid degradation	25.3mg Ondansetron bulk drug, and 5 ml 5.0M HCl. Heated at 100°C for 10 min.
Alkali degradation	25.2mg Ondansetron bulk drug,and 5ml 5M NaOH. Heated at 100°C for 10 min.
Peroxide degradation	25.1mg Ondansetron bulk drug, and 5 ml of 30.0% H_2O_2. Heated at 100°C for 10 min.
Thermal degradation	24.7mg Ondansetron bulk drug and 20ml mobile phase. Heated at 100°C for 10 min.

Acid and Alkali degradation pH was adjusted to 7.0 before injection into the system.

5. Linearity Study

The linearity of detector (UV) response for impurities was determined by preparing and injecting solutions in the concentration range of 0.5 –1.5µg/ml(impurity A and C) and 0.25-0.75µg/ml (impurity D) i.e(50-150% of limit conc.) of Ondansetron standard.

Stock Solution

Solution A

Transfer 12.51 mg, 12.43 mg, each of impurities A and C accurately weighed, to a 25 ml volumetric flask Containing 10 ml of mobile phase. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution B

Transfer 12.55 mg of impurity D, accurately weighed, to a 50 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution C

5 ml of solution A and 5 ml of solution B is diluted to 100 ml by mobile phase. This solution was diluted as shown in Table - 4.2.8c

Table 4.2.8c: Solution preparation for linearity study of Ondansetron impurities

% level of std	Vol. of stock solution C (ml)	Final Dil (ml)	Final concentrations (µg/ml)		
			Imp A	Imp C	Imp D
50	1.0	50	0.50	0.50	0.25
75	1.5	50	0.75	0.75	0.38
100	2.0	50	1.00	0.99	0.50
125	2.5	50	1.25	1.24	0.63
150	3.0	50	1.50	1.49	0.75

Ondansetron stock solution

The linearity of detector (UV) response for ondansetron was determined by preparing and injecting solutions in the concentration range of 500 –0.25μg/ml.(for area normalization calculation of unknown impurities).

Table 4.2.8d : Solution preparation for linearity study of Ondansetron bulk drugs

% level of std conc	Std weight (mg)	Final dilutions (μg/ml)					Concentration of ondansetron
		1st dilution	2nd dilution		3rd dilution		
0.05	25.75	50	2	100	2.5	100	0.26
0.20	25.75	50	2	100	5	50	1.03
2.0	25.75	50	2	100	1	1	10.3
20.0	25.75	50	2	10	1	1	103
100.0	25.75	50	1	1	1	1	515

6. Method Precision

Stock solution

Solution A

Transfer 12.51 mg, 12.43 mg, each of impurities A and C accurately weighed, to a 25 ml volumetric flask Containing 10 ml of mobile phase. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution B

Transfer 12.55 mg of impurity D, accurately weighed, to a 50 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution C

Transfer 12.39 mg of ondansetron, accurately weighed, to a 25 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution D

5 ml each of solution A ,B and C was pipetted out into a 100 ml volumetric flask and diluted by mobile phase upto the mark.

Solution E

5 ml each of solution A and B was pipetted out into a 100 ml volumetric flask and diluted by mobile phase upto the mark.

Standard preparation

Transfer about 2 ml of solution D into a 50 ml volumetric flask. Dissolve in and dilute up to mark with mobile phase. Above mentioned solution was injected six times and mean, % RSD of individual impurities and Ondansetron were observed.

Sample preparation

Six sets of Ondansetron were prepared as per the method on same day for analysis by the HPLC method . Table 4.2.8e shows solution preparation for method precision sets.

Table 4.2.8e : Solution preparation for method precision sets of Ondansetron Bulk drug

Wt. of std (mg)	Vol. of stock solution E (ml)	Final Dil. (ml)	Final concentrations (μg/ml)			
			Imp A	Imp C	Imp D	Ondansetron
25.57	2.0	50	1.00	0.99	0.50	511.4
25.55	2.0	50	1.00	0.99	0.50	511.0
25.26	2.0	50	1.00	0.99	0.50	505.2
25.01	2.0	50	1.00	0.99	0.50	500.2
26.09	2.0	50	1.00	0.99	0.50	521.8
26.12	2.0	50	1.00	0.99	0.50	522.4

7. Recovery(Accuracy)

Recovery study was performed by spiking impurities in the Ondansetron standard at levels 70%, 85%,100%,115% & 130 % of limit concentration(0.2% for imp A,C and 0.1% for imp D). The samples were chromatographed in triplicate according to the procedure. Table 4.2.7f shows solution preparation for recovery study.

Stock solution

Solution A
Transfer 12.51 mg, 12.43 mg, each of impurities A and C accurately weighed, to a 25 ml volumetric flask Containing 10 ml of mobile phase. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution B
Transfer 12.55 mg of impurity D, accurately weighed, to a 50 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution C
Transfer 12.39 mg of ondansetron, accurately weighed, to a 25 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution D
5 ml each of solution A, B and C was pipetted out into a 100 ml volumetric flask and diluted by mobile phase upto the mark.

Solution E
5 ml each of solution A and B was pipetted out into a 100 ml volumetric flask and diluted by mobile phase upto the mark.

Standard preparation

Transfer about 2 ml of solution D into a 50 ml volumetric flask. Dissolve in and dilute up to mark with mobile phase. Above mentioned solution was injected six times and mean, % RSD of individual impurities and Ondansetron were observed.

Table 4.2.8f : Solution preparation for recovery study of Ondansetron bulk drug

% level std	wt std (mg)	Vol of stock sol D (ml)	Final Dil. (ml)	Final Concentrations (µg/ml)			
				Imp A	Imp C	Imp D	Ondansetron
70	25.25	1.4	50	0.70	0.70	0.35	505.0
85	25.14	1.7	50	0.85	0.85	0.43	502.8
100	25.06	2.0	50	1.00	0.99	0.50	501.2
115	25.27	2.3	50	1.15	1.14	0.58	505.4
130	25.31	2.6	50	1.30	1.29	0.65	506.2

8. Limit of detection and Limit of quantitation

For determining the limit of detection (LOD) and limit of quantitation (LOQ), the method based on the residual standard deviation of a regression line and slope was adopted.

Stock Solution

Solution A

Transfer 12.51 mg, 12.43 mg, each of impurities A and C accurately weighed, to a 25 ml volumetric flask Containing 10 ml of mobile phase. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution B
Transfer 12.55 mg of impurity D, accurately weighed, to a 50 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution C
5 ml each of solution A and B was pipetted out into a 100 ml volumetric flask and diluted by mobile phase upto the mark.

Solution D
1 ml of solution C is diluted to 50 ml by mobile phase.

Table 4.2.8g : Solution preparation for Limit of detection and quantitation study of Ondansetron

Sol No.	Vol. of stock sol D (ml)	Final Dil (ml)	Final concentrations (μg/ml)		
			ImpA	ImpC	ImpD
1	1	1	0.500	0.497	0.251
2	5	10	0.250	0.249	0.126
3	2.5	10	0.125	0.124	0.063
4	1.2	10	0.060	0.060	0.030
5	0.6	10	0.030	0.030	0.015
6	0.3	10	0.015	0.015	0.008
7	0.1	10	0.005	0.005	0.003

9. Ruggedness

Method ruggedness was established by analysing sample at normal operating condition and at changed conditions then results were compared. Table no.4.2.8i shows details of solution preparation for ruggedness study.

Stock Solution

Solution A
Transfer 12.51 mg, 12.43 mg, each of impurities A and C accurately weighed, to a 25 ml volumetric flask Containing 10 ml of mobile phase. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution B

Transfer 12.55 mg of impurity D, accurately weighed, to a 50 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution C

Transfer 12.39 mg of ondansetron, accurately weighed, to a 25 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution D

5 ml each of solution A, B and C was pipetted out into a 100 ml volumetric flask and diluted by mobile phase upto the mark.

Solution E

5 ml each of solution A and B was pipetted out into a 100 ml volumetric flask and diluted by mobile phase upto the mark.

Table 4.2.8h : Solution preparation for ruggedness study of Ondansetron Bulk drug

Parameter Changed	Condition	Solution	Wt (mg)	Vol. of stock D (ml)	Vol. of stock E (ml)	Final Dilution (ml)
Original	As described in method	Standard	-	2	-	50
		Sample	25.15	-	2	50
Analyst	Analysis performed by another analyst, other conditions constant	Standard	-	2	-	50
		Sample	25.01	-	2	50
Column	Thermoquest, Hypersil (C8,250 *4.6 mm,5 μ) other conditions constant	Standard	-	2	-	50
		Sample	25.17	-	2	50
Flow rate changed	Flow rate was changed to 1.5ml/min.other conditions constant	Standard	-	2	-	50
		Sample	25.24	-	2	50
Mobile phase Com position	Buffer: acetonitrile::69:31 other conditions constant	Standard	-	2	-	50
		Sample	25.23	-	2	50
Instrument change	Instrument configuration was changed. other conditions constant	Standard	-	2	-	50
		Sample	25.24	-	2	50

10. Stability of analytical solution

Stock Solution

Solution A
Transfer 12.51 mg, 12.43 mg, each of impurities A and C accurately weighed, to a 25 ml volumetric flask Containing 10 ml of mobile phase. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution B
Transfer 12.55 mg of impurity D, accurately weighed, to a 50 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution C
Transfer 12.39 mg of ondansetron, accurately weighed, to a 25 ml volumetric flask. After sonication for 10 min. dissolve in and dilute up to mark with mobile phase.

Solution D
2 ml of solution A, C and 4 ml of solution B is diluted to 10 ml by mobile phase.

Solution D was injected in duplicate at different time intervals and peak areas were recorded.

CHAPTER – 5

RESULT AND DISCUSSION

The HPLC methods developed and presented were aimed at developing a chromatographic system capable of resolving the drug peak from all of its degradation product peaks.

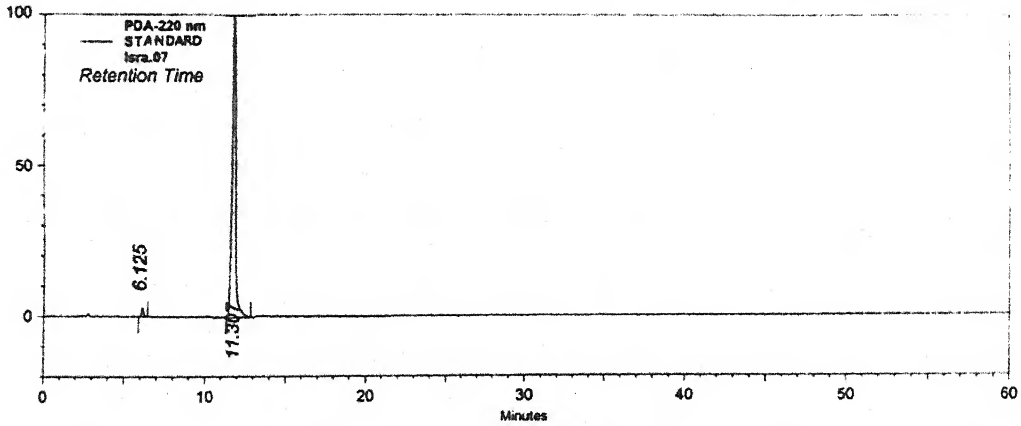
The preliminary investigations were directed towards the effect of various variables on the system suitability of the method. The parameters assessed include the detection wavelength, the type and quantity of organic modifier, the column, concentration of buffer, flow rate and the pH of the mobile phase. The analytical wavelength was finalized by taking into consideration of low interference of solvent , placebo and optimum response of the drug and degradation products at various wavelength. Column for the method was finalized by having maximum no. of theoretical plates, least tailing, best peak shape, good separation and less runtime. In choosing mobile phase importance is given to achieve baseline separation of the drug and its degradation products. pH effect on buffer was also studied to achieve good separation between peaks and does not effect the separation even if there is slight deviation in the pH. Above all due importance is given on the total cost involved, as the methods are to be used for routine analysis, stability analysis where large number of samples are to analysed. Priority is given to choose the materials which are easily available and low cost, where as quality of the analyis is not affected.

Consequently, the optimum condition mentioned under section experiment were applied and the method were subjected to validations according to the ICH guidelines. The validation data obtained for each drug have been discussed.

5.1 Candesartan

The retention time of candesartan peak was about 11.3 min. The relative standard deviation of the area of the candesartan peak for replicate injections was found to be less than 0.18%.The column efficency was measured by no. of theoretical plates which was found to be greater than 8000 and the tailing factor of 1.17.Typical chromatograph of candesartan is shown in figure 5.1F1.

Figure 5.1F1 : Typical chromatograph of Candesartan

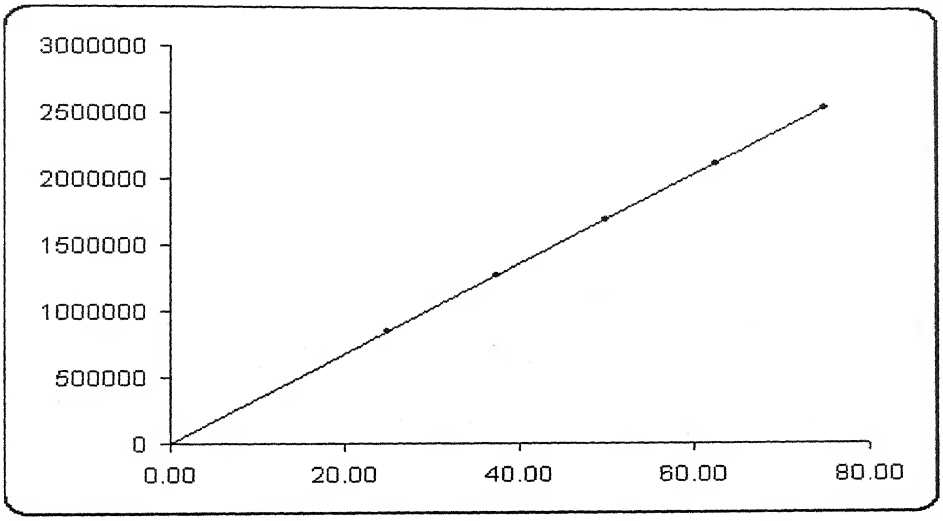


Method precision shows a mean of 93.64% label claim with a RSD of 0.59% was obtained. The mean recovery data for each level is within accepted values (100.22, 99.03 and 98.26 % label claim for 80,100 and 120%level respectively).Therefore, these results indicated a good accuracy of the method for Candesertan. The mean recovery was 99.17 % label claim and % RSD was 0.99.

The method was shown to be linear from 25.04 to 75.12 µg/ml of candesartan concentration. A calibration curve was constructed using characteristic

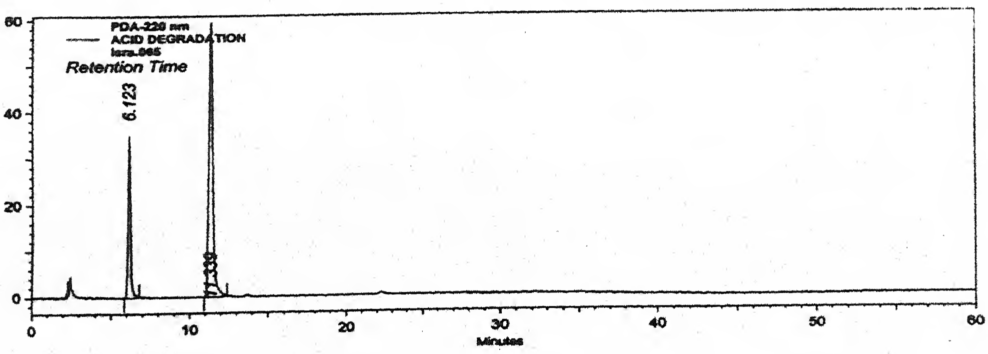
parameters for regression equation (Y= a + bx) and coefficient of correlation r^2 was found to be 1.000. The linearity graph is shown in figure 5.1F2.

Figure 5.1F2 : Linearity graph of Candesartan

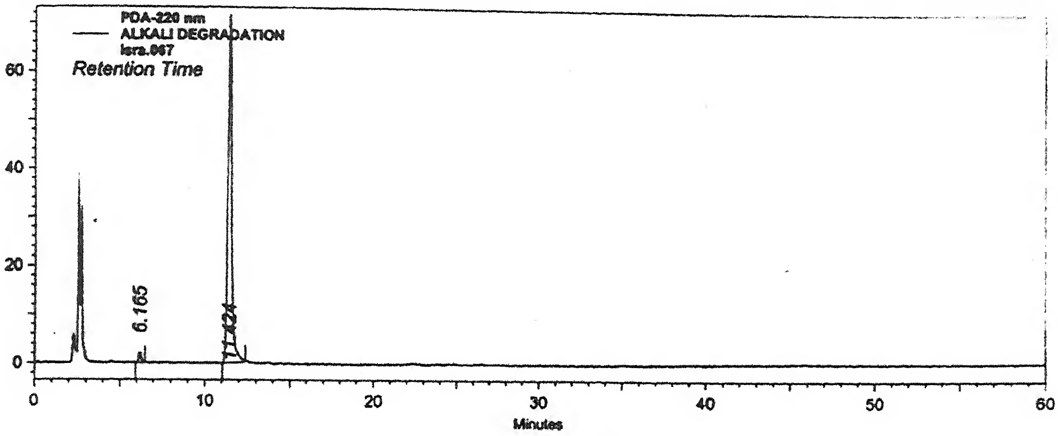


All the degradation peaks generated in the forced degradation studies were well separated and peak purity of candesartan peak was always greater than 99.0 % proving the stability indicating nature of the method. Major degradation was observed under acidic, alkaline and oxidative degradation conditions. Typical chromatographs obtained in degradation study are shown in figure 5.1F3.

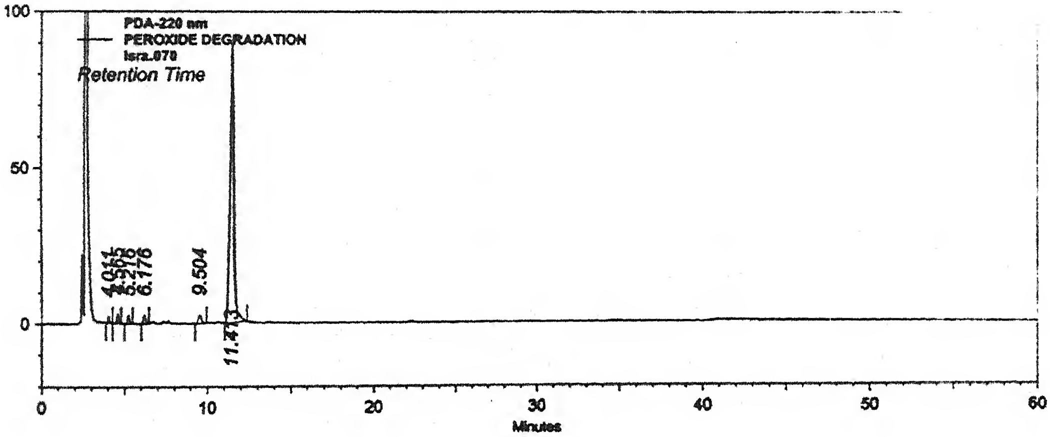
Figure 5.1F3 : Specificity study chromatographs of Candesartan



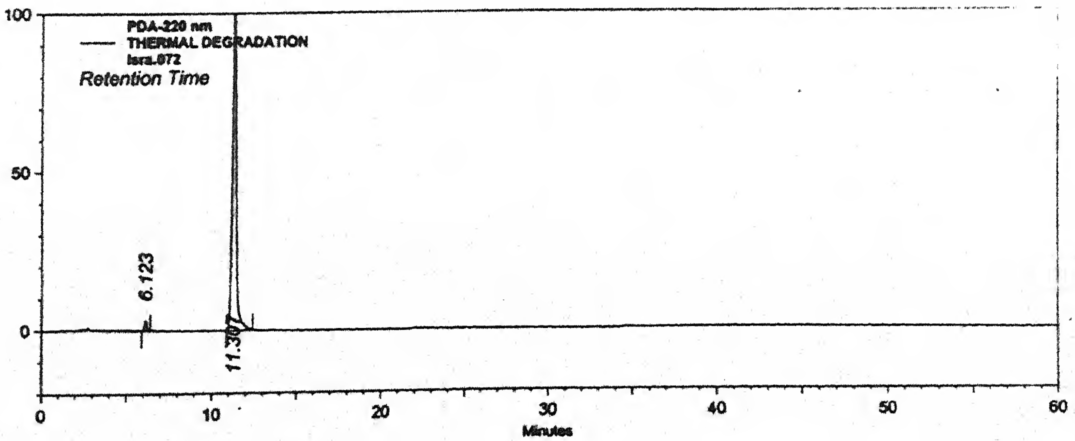
Acid degradation



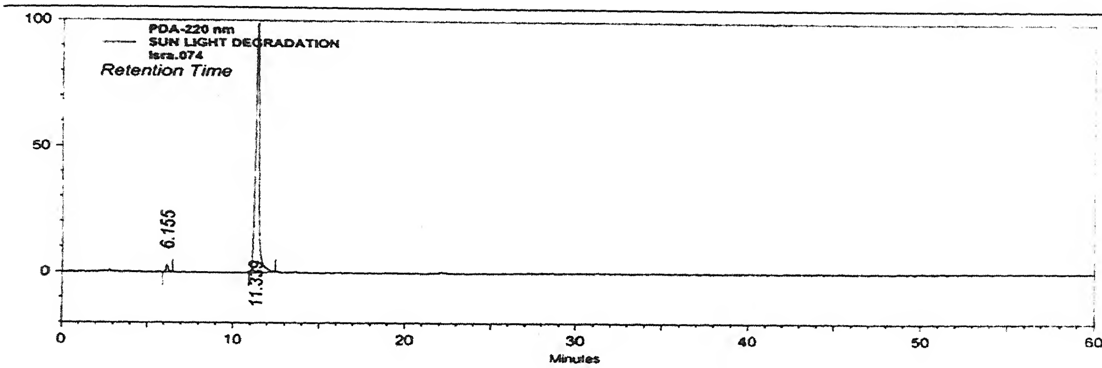
Alkali degradation



Peroxide degradation



Thermal degradation



Sun-light degradation

The result obtained from degradation study shows peak purity of Candesertan was 100 % as calculated by PDA detector, proving that no degradation product is interfering with the main peak. The % residual drug was calculated in comparison with the standard, which is 55.71, 62.16, 79.89, 91.15 and 86.87% for Acid, Alkali, Peroxide, Thermal and sunlight degradation respectively.

The result obtained during robustness shows that by changing deliberately, some internal and external parameters of the method does not influence the results. solution stability of 14 hrs was observed.

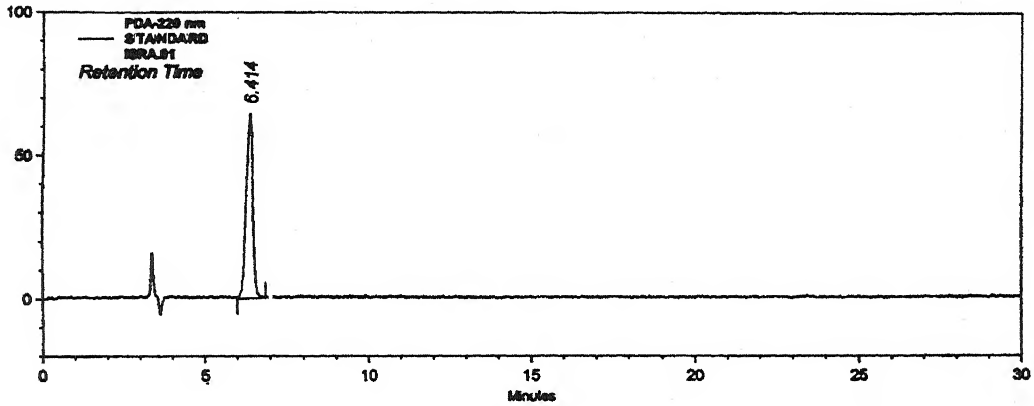
Table 5.1.1 : Summary of the performance parameters of the HPLC procedure for Candesertan Tablets

S.no	Parameters	Observed value
1.	System Suitability	
	a.Theoretical Plates	8155
	b. Tailing Factor	1.17
2.	Instrument Precision	RSD 0.18 %
3.	Method Precision	Label claim 93.64 %
4.	Linearity and range	Correlation coefficient(r^2) = 1.0000
5.	Accuracy	Mean recovery 99.17%
6.	Specificity	Peak Purity of candesertan peak after degradation was 100.0 %
7.	Robustness	Difference from original condition 0.44%
8.	Solution stability	14 hrs

5.2 Captopril

The retention time of Captopril peak was about 6.4 min. The relative standard deviation of the area of the Captopril peak for replicate injections was found to be less than 0.61%.The column efficency was measured by no. of theoretical plates which was found to be greater than 5400 and the tailing factor of 0.99.Typical chromatograph of Captopril is shown in figure 5.2F1.

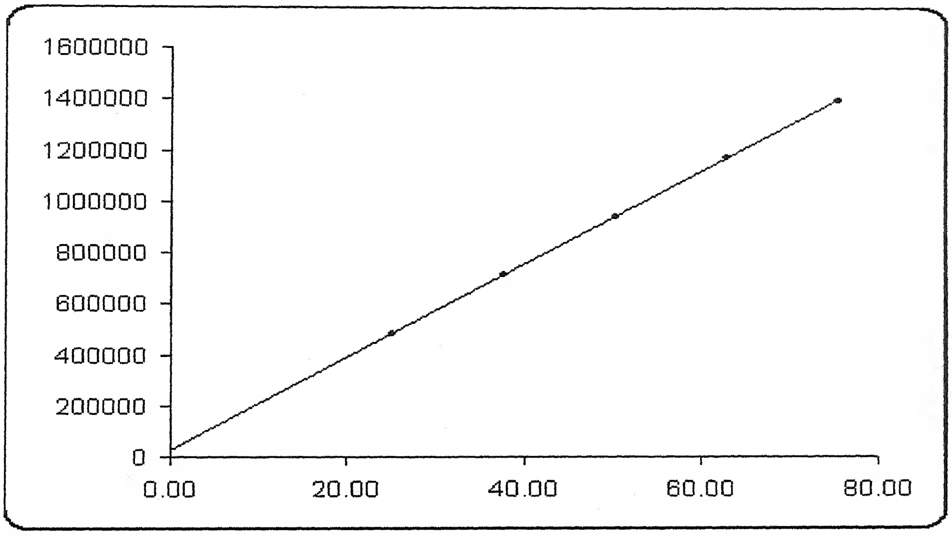
Figure 5.2 F1 : Typical chromatograph of Captopril



Method precision shows a mean of 100.19 % label claim with a RSD of 0.43% was obtained. The mean recovery data for each level is within accepted values (99.93, 99.17 and 99.20 % label claim for 80,100 and 120%level respectively).Therefore, these results indicated a good accuracy of the method for Captopril. The mean recovery was 99.43 % label claim and % RSD was 0.43.

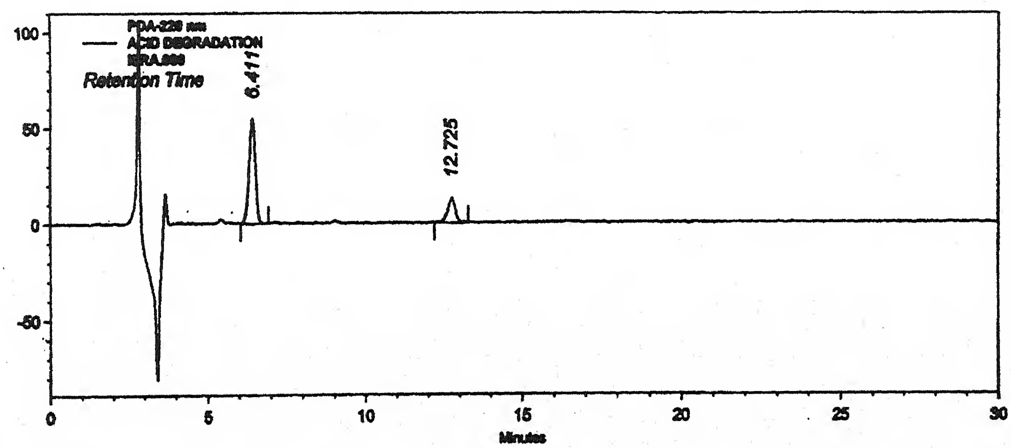
The method was shown to be linear from 25.12 to 75.36 µg/ml of Captopril concentration. A calibration curve was constructed using characteristic parameters for regression equation (Y= a + bx) and coefficient of correlation r^2 was found to be 1.000.The linearity graph is shown in figure 5.2F2.

Figure 5.2F2 : Linearity graph of Captopril

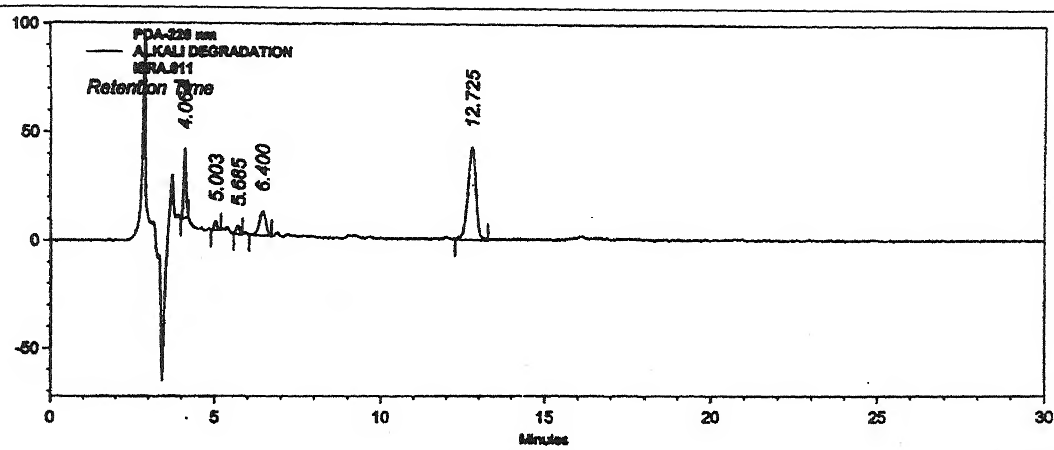


All the degradation peaks generated in the forced degradation studies were well separated and peak purity of Captopril peak was always greater than 99.0 % proving the stability indicating nature of the method. Major degradation was observed under acidic, alkaline and oxidative degradation conditions. Typical chromatographs obtained in degradation study are shown in figure 5.2F3.

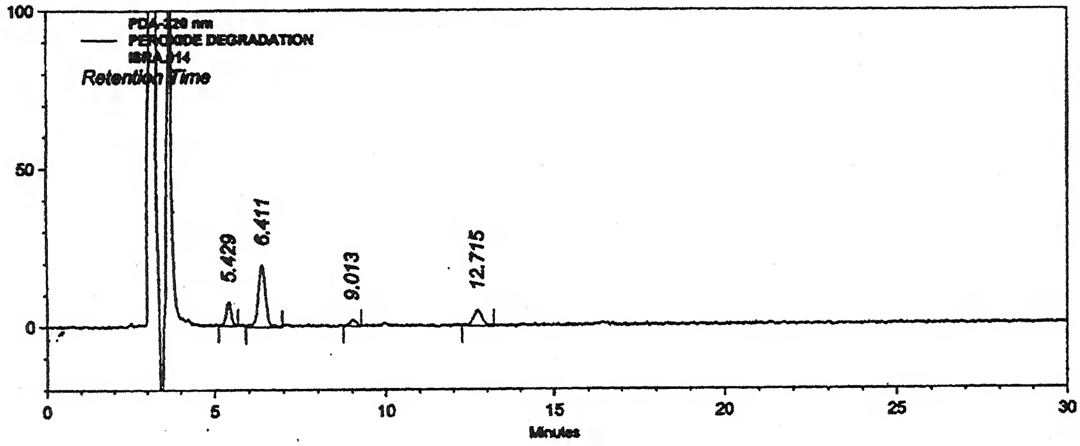
Figure 5.2F3 : Specificity study chromatographs of Captopril



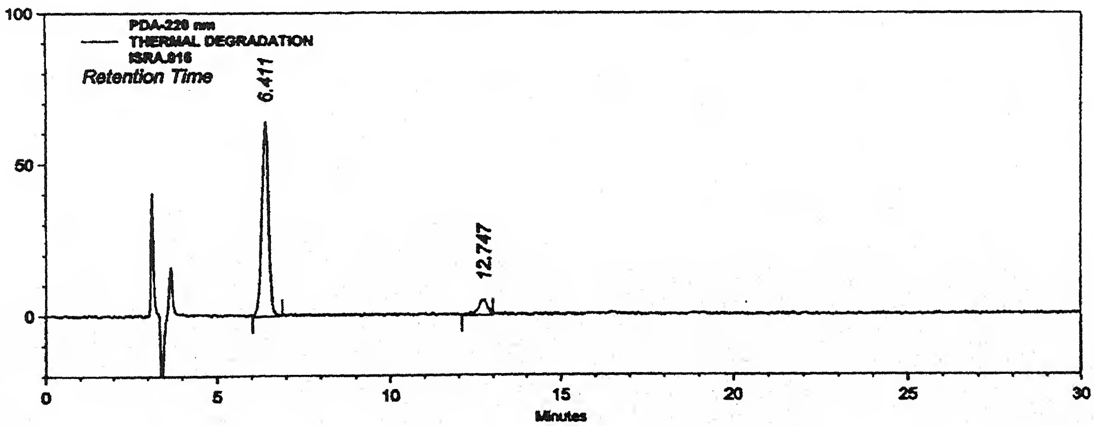
Acid degradation



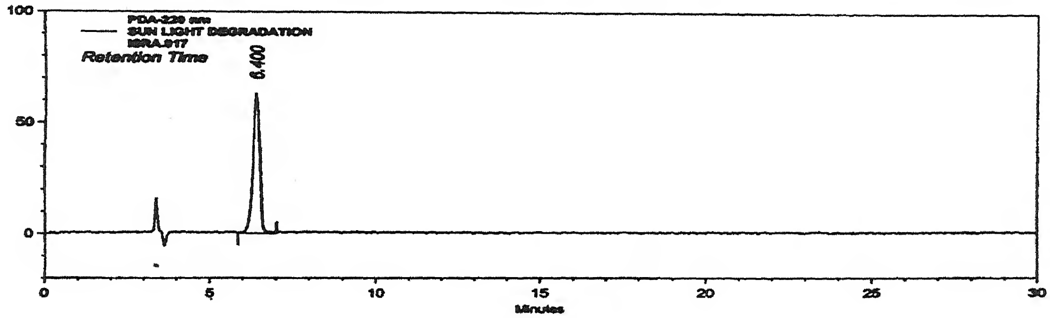
Alkali degradation



Peroxide degradation



Thermal degradation



Sun-light degradation

The result obtained from degradation study shows peak purity of Captopril was 100 % as calculated by PDA detector, proving that no degradation product is interfering with the main peak. The % residual drug was calculated in comparison with the standard, which is 79.91, 17.30, 30.91, 95.86 and 97.06% for Acid, Alkali, Peroxide, Thermal and sunlight degradation respectively.

The result obtained during robustness shows that by changing deliberately, some internal and external parameters of the method does not influence the results. solution stability of 19 hrs was observed.

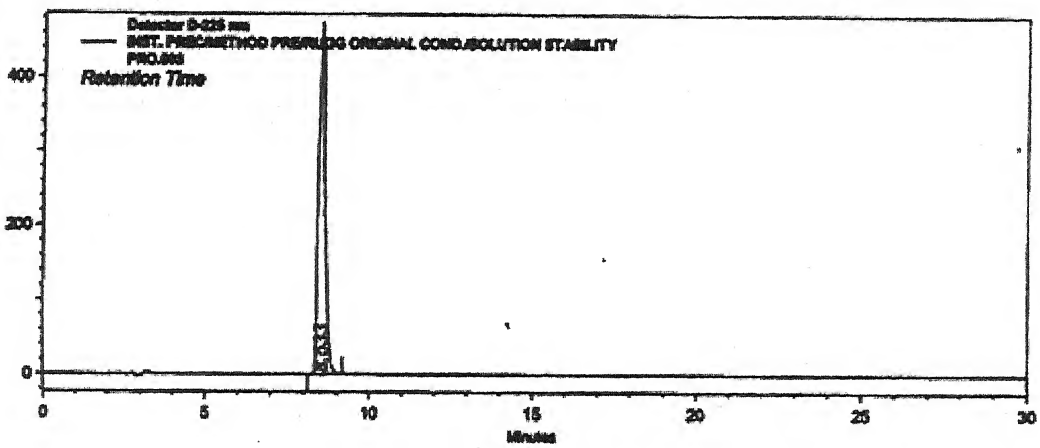
Table 5.2.1 : Summary of the performance parameters of the HPLC procedure for Captopril Tablets

S.no	Parameters	Observed value
1.	System Suitability	
	a.Theoretical Plates	5400
	b. Tailing Factor	0.99
2.	Instrument Precision	RSD 0.61 %
3.	Method Precision	Label claim 100.19 %
4.	Linearity and range	Correlation coefficient(r^2) = 1.0000
5.	Accuracy	Mean recovery 99.43%
6.	Specificity	Peak Purity of Captopril peak after degradation was 100.0 %
7.	Robustness	Difference from original condition 0.44%
8.	Solution stability	19 hrs

5.3 Propranolol

The retention time of Propranolol peak was about 8.5 min. The relative standard deviation of the area of the Propranolol peak for replicate injections was found to be less than 0.19%.The column efficency was measured by no. of theoretical plates which was found to be greater than 8000 and the tailing factor of 1.31.Typical chromatograph of Propranolol is shown in figure 5.3F1.

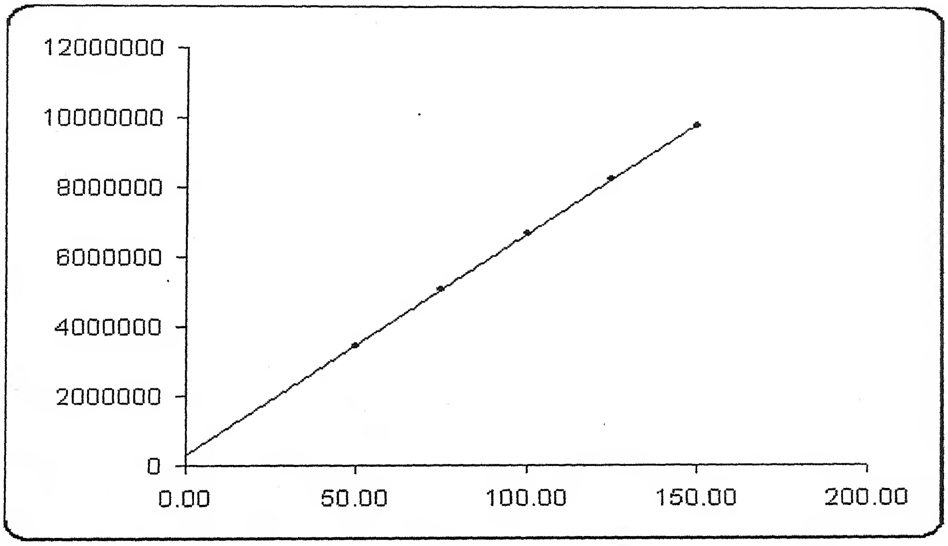
Figure 5.3F1 : Typical chromatograph of Propranolol



Method precision shows a mean of 100.43% label claim with a RSD of 0.48% was obtained. The mean recovery data for each level is within accepted values (101.4, 100.7 and 99.4%label claim for 80,100 and 120%level respectively).Therefore, these results indicated a good accuracy of the method for Propranolol. The mean recovery was 100.5 % label claim and % RSD was 1.03.

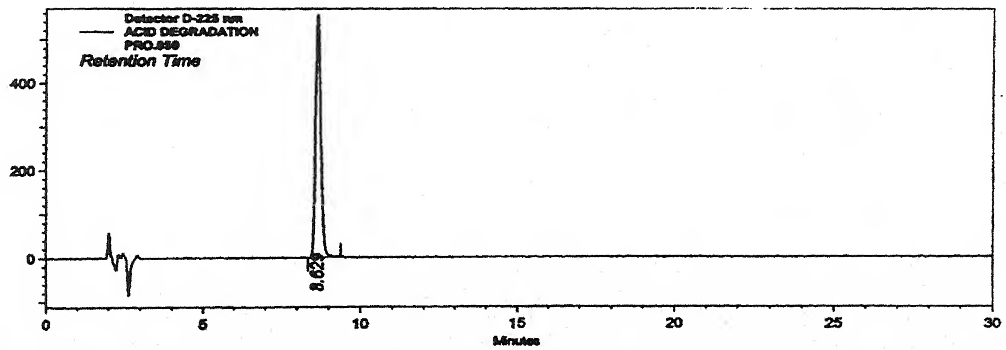
The method was shown to be linear from 50.1 to 150.2 µg/ml of Propranolol concentration. A calibration curve was constructed using characteristic parameters for regression equation (Y= a + bx) and coefficient of correlation r^2 was found to be 1.000.The linearity graph is shown in figure 5.3F2.

Figure 5.3F2 : Linearity graph of Propranolol

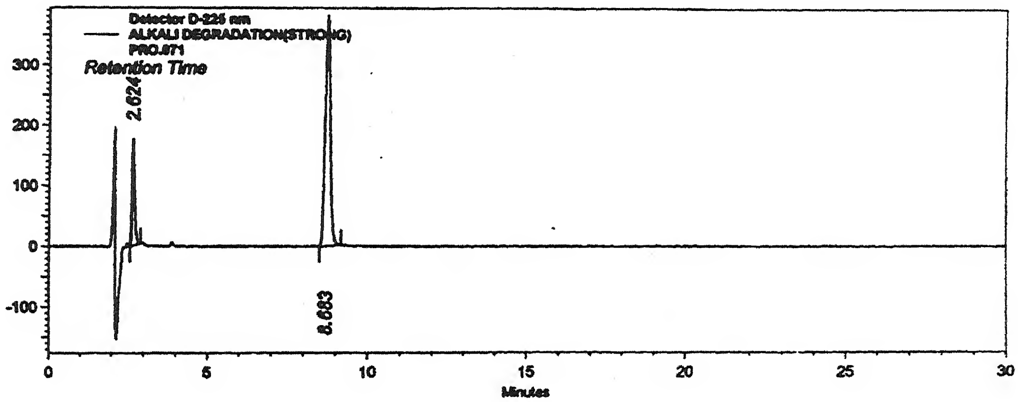


All the degradation peaks generated in the forced degradation studies were well separated and peak purity of Propranolol peak was always greater than 99.0 % proving the stability indicating nature of the method. Major degradation was observed under all studied degradation conditions. Typical chromatographs obtained in degradation study are shown in figure 5.3F3.

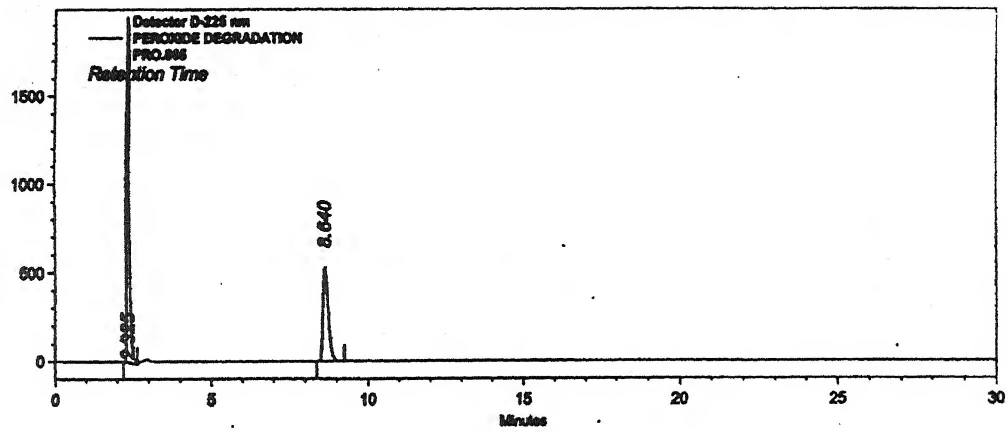
Figure 5.3F3 : Specificity study chromatographs of Propranolol



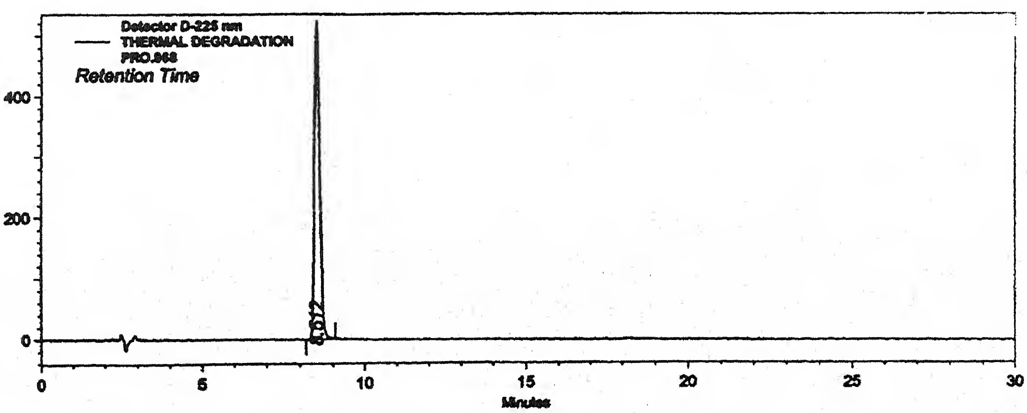
Acid degradation



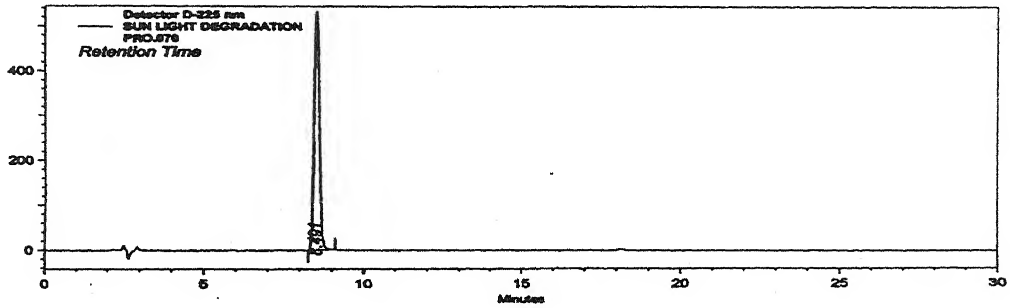
Alkali degradation



Peroxide degradation



Thermal degradation



Sun-light degradation

The result obtained from degradation study shows peak purity of Propranolol was 100 % as calculated by PDA detector, proving that no degradation product is interfering with the main peak. The % residual drug was calculated in comparison with the standard, which is 88.6, 61.1, 86.4, 85.0 and 86.9% for Acid, Alkali, Peroxide, Thermal and sunlight degradation respectively.

The result obtained during robustness shows that by changing deliberately, some internal and external parameters of the method does not influence the results. solution stability of 27 hrs was observed.

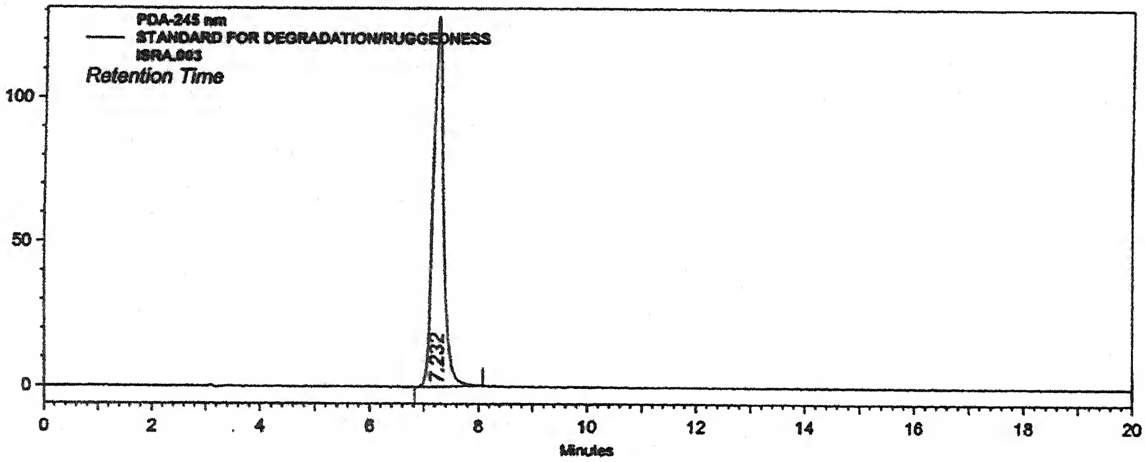
Table 5.3.1 : Summary of the performance parameters of the HPLC procedure for Propranolol Tablets

S.no	Parameters	Observed value
1.	System Suitability a.Theoretical Plates b. Tailing Factor	 8134 1.31
2.	Instrument Precision	RSD 0.19 %
3.	Method Precision	Label claim 100.43 %
4.	Linearity and range	Correlation coefficient(r^2) = 1.0000
5.	Accuracy	Mean recovery 100.51%
6.	Specificity	Peak Purity of propranolol peak after degradation was 100.0 %
7.	Robustness	Difference from original condition 0.15%
8.	Solution stability	27 hrs

5.4 Terazosin

The retention time of Terazosin peak was about 7.0 min. The relative standard deviation of the area of the Terazosin peak for replicate injections was found to be less than 0.64%.The column efficency was measured by no. of theoretical plates which was found to be greater than 7000 and the tailing factor of 1.22.Typical chromatograph of Terazosin is shown in figure 5.4F1.

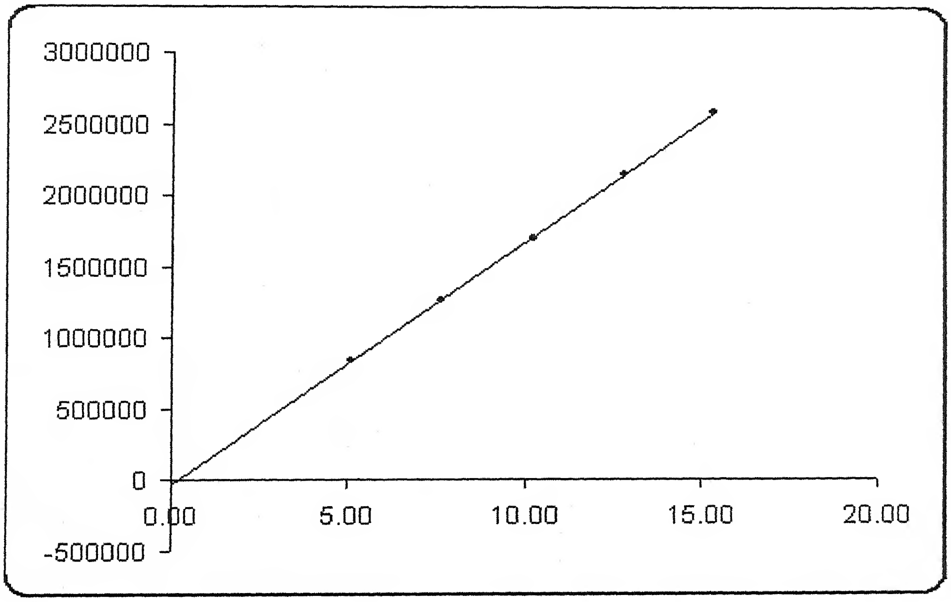
Figure 5.4F1 : Typical chromatograph of Terazosin



Method precision shows a mean of 102.00% label claim with a RSD of 0.62% was obtained. The mean recovery data for each level is within accepted values (100.2, 98.6 and 99.8%label claim for 80,100 and 120%level respectively).Therefore, these results indicated a good accuracy of the method for Terazosin. The mean recovery was 99.5 % label claim and % RSD was 0.81.

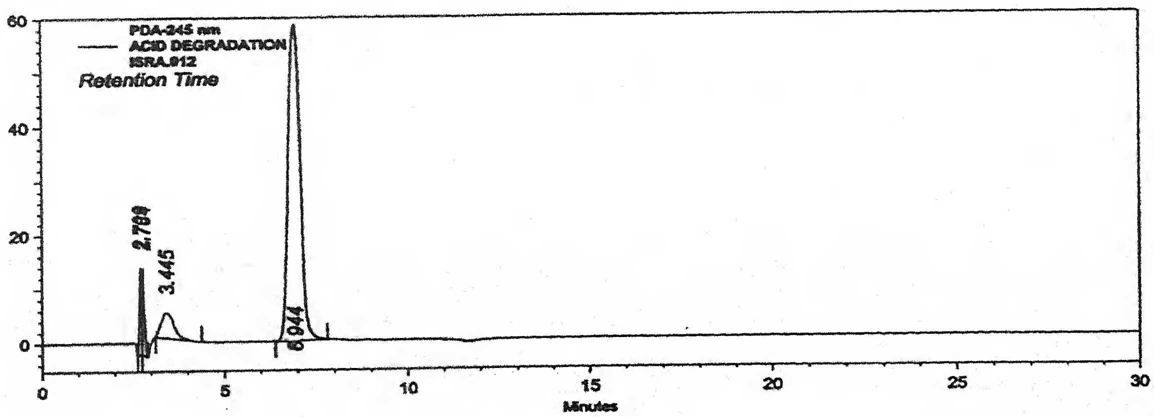
The method was shown to be linear from 5.1 to 15.3 µg/ml of Terazosin concentration. A calibration curve was constructed using characteristic parameters for regression equation (Y= a + bx) and coefficient of correlation r^2 was found to be 1.000.The linearity graph is shown in figure 5.4F2.

Figure 5.4F2 : Linearity graph of Terazosin

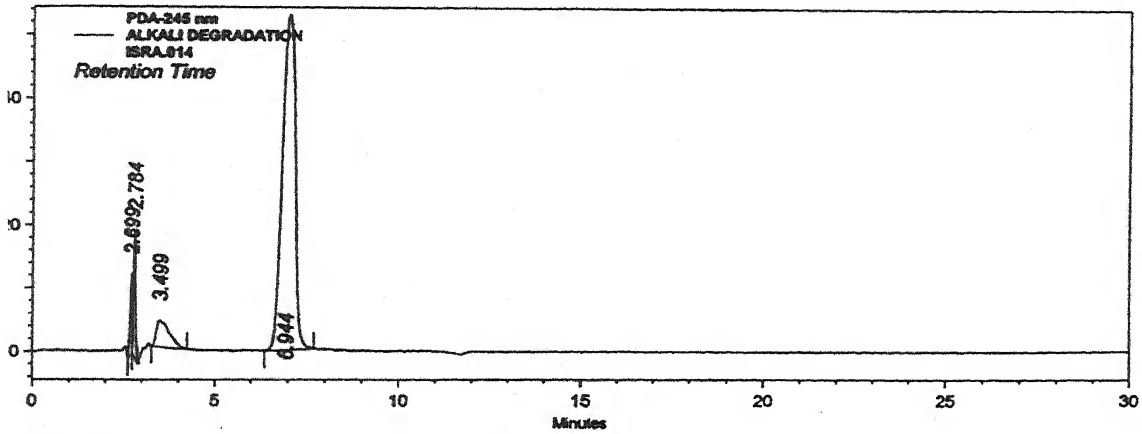


All the degradation peaks generated in the forced degradation studies were well separated and peak purity of Terazosin peak was always greater than 99.0 % proving the stability indicating nature of the method. Major degradation was observed under all studied degradation conditions. Typical chromatographs obtained in degradation study are shown in figure 5.4F3.

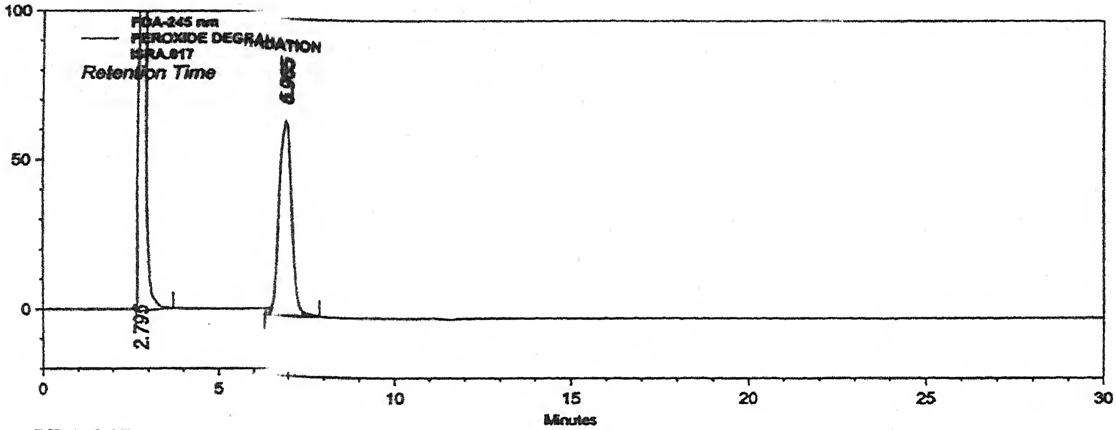
Figure 5.4F3 : Specificity study chromatographs of Terazosin



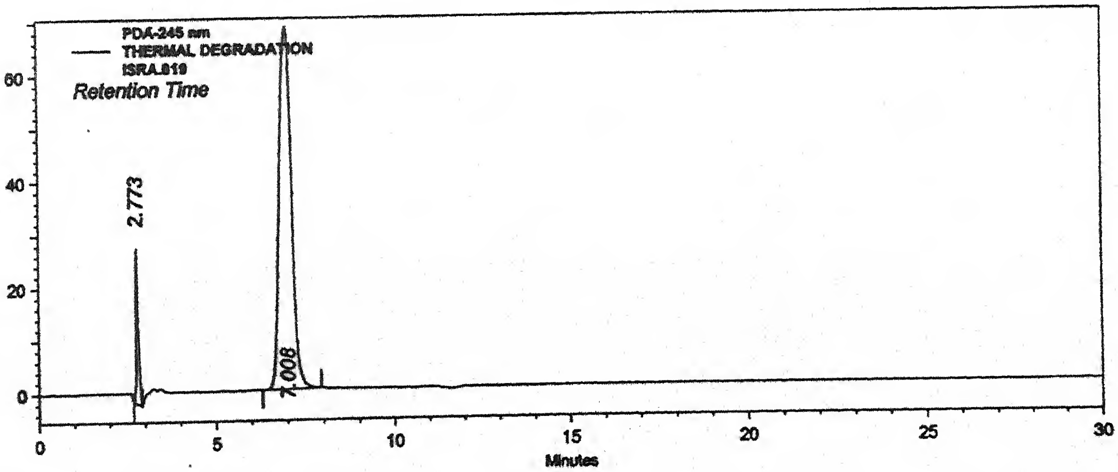
Acid degradation



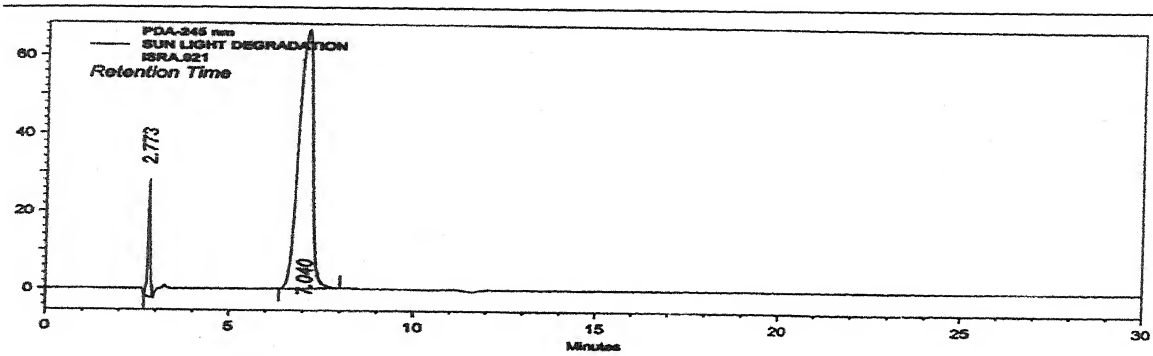
Alkali degradation



Peroxide degradation



Thermal degradation



Sun-light degradation

The result obtained from degradation study shows peak purity of Terazosin was 100 % as calculated by PDA detector, proving that no degradation product is interfering with the main peak. The % residual drug was calculated in comparison with the standard, which is 78.1, 75.9, 94.6, 94.9 and 97.5% for Acid, Alkali, Peroxide, Thermal and sunlight degradation respectively.

The result obtained during robustness shows that by changing deliberately, some internal and external parameters of the method does not influence the results. solution stability of 18 hrs was observed.

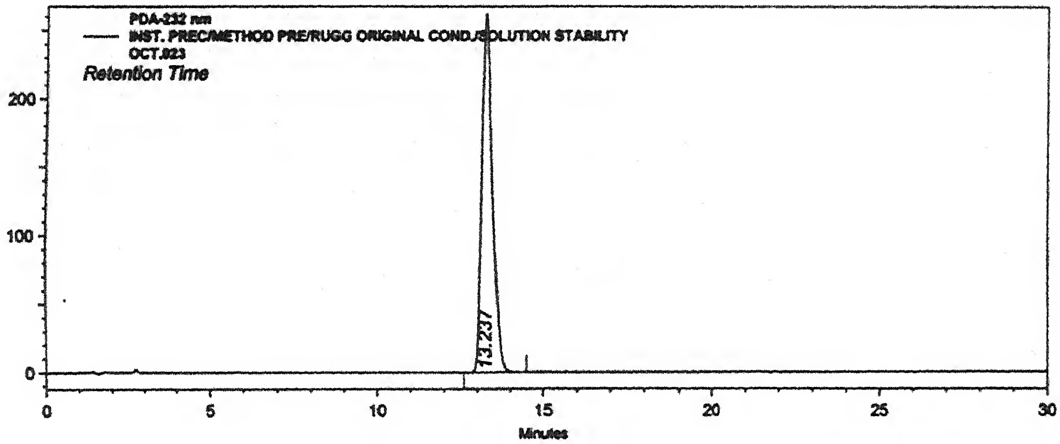
Table 5.4.1 : Summary of the performance parameters of the HPLC procedure for Terazosin Tablets

S.no	Parameters	Observed value
1.	System Suitability	
	a.Theoretical Plates	7339
	b. Tailing Factor	1.22
2.	Instrument Precision	RSD 0.64 %
3.	Method Precision	Label claim 102.00 %
4.	Linearity and range	Correlation coefficient(r^2) = 1.0000
5.	Accuracy	Mean recovery 99.52%
6.	Specificity	Peak Purity of Terazosin peak after degradation was 100.0 %
7.	Robustness	Difference from original condition 0.20%
8.	Solution stability	18 hrs

5.5 Verapamil

The retention time of Verapamil peak was about 13.2 min. The relative standard deviation of the area of the Verapamil peak for replicate injections was found to be less than 0.27%.The column efficency was measured by no. of theoretical plates which was found to be greater than 8000 and the tailing factor of 1.55.Typical chromatograph of Verapamil is shown in figure 5.5F1.

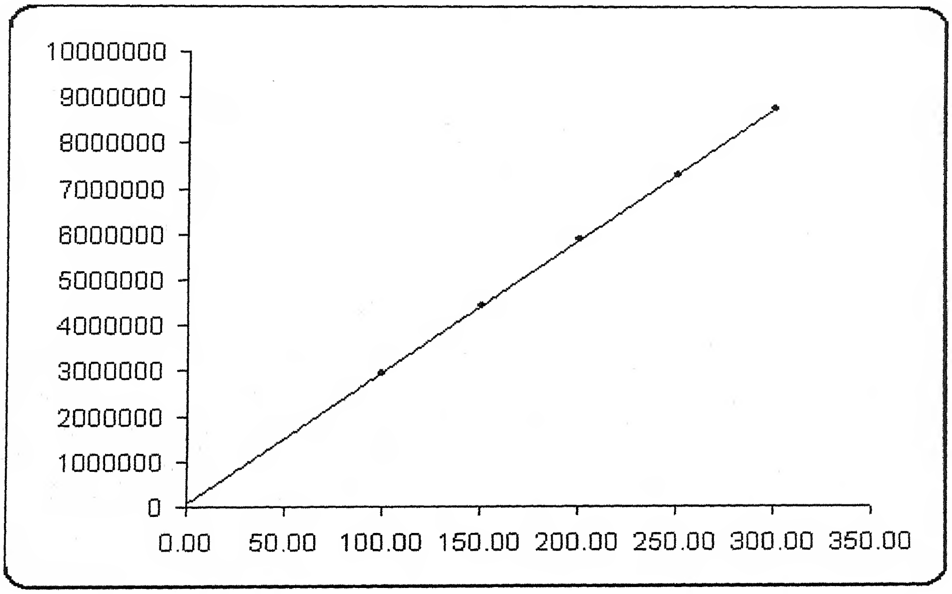
Figure 5.5F1 : Typical chromatograph of Verapamil



Method precision shows a mean of 103.3% label claim with a RSD of 0.24% was obtained. The mean recovery data for each level is within accepted values (101.0, 101.3 and 98.6%label claim for 80,100 and 120%level respectively).Therefore, these results indicated a good accuracy of the method for Verapamil. The mean recovery was 100.3 % label claim and % RSD was 1.5.

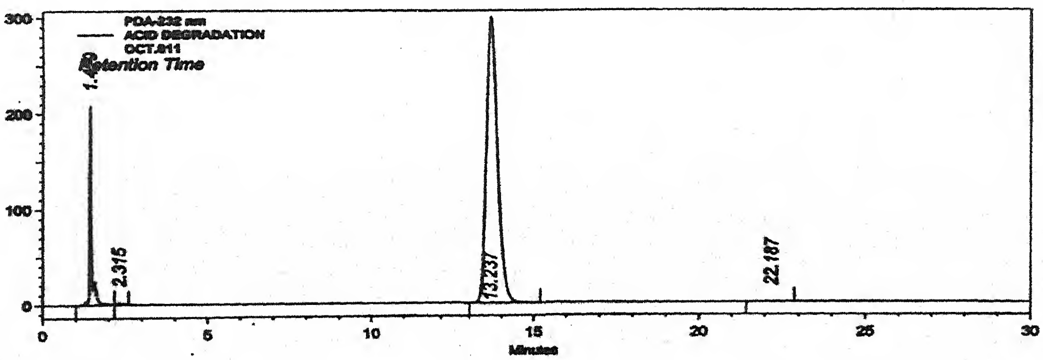
The method was shown to be linear from 100.5 to 301.4 µg/ml of Verapamil concentration. A calibration curve was constructed using characteristic parameters for regression equation (Y= a + bx) and coefficient of correlation r^2 was found to be 1.000.The linearity graph is shown in figure 5.5F2.

Figure 5.4F2 : Linearity graph of Verapamil

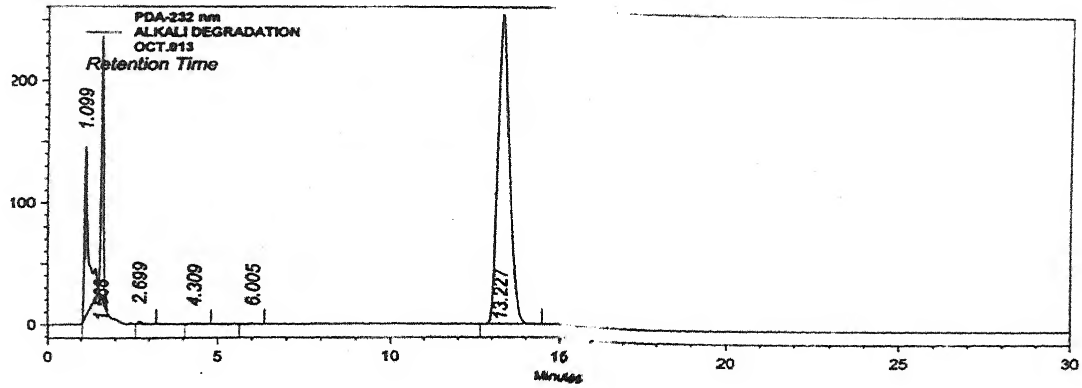


All the degradation peaks generated in the forced degradation studies were well separated and peak purity of Verapamil peak was always greater than 99.0 % proving the stability indicating nature of the method. Major degradation was observed under Alkali and Peroxide degradation conditions. Typical chromatographs obtained in degradation study are shown in figure 5.5F3.

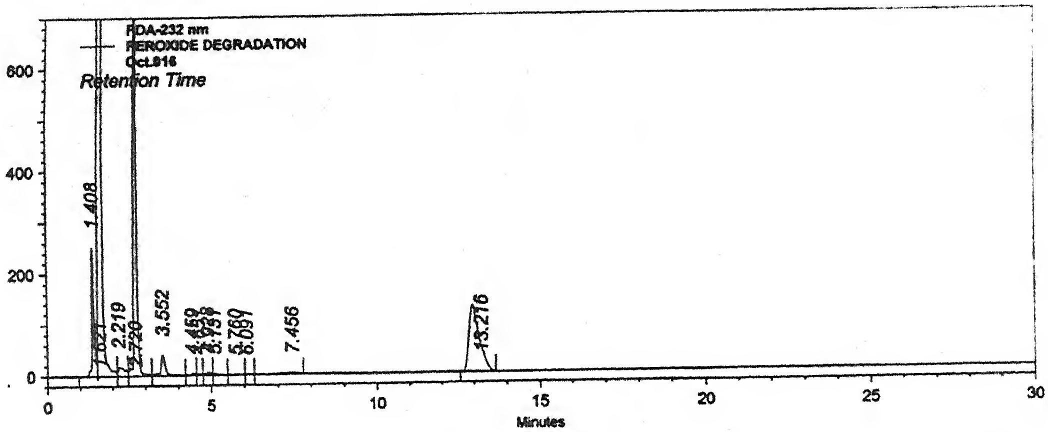
Figure 5.5F3 : Specificity study chromatographs of Verapamil



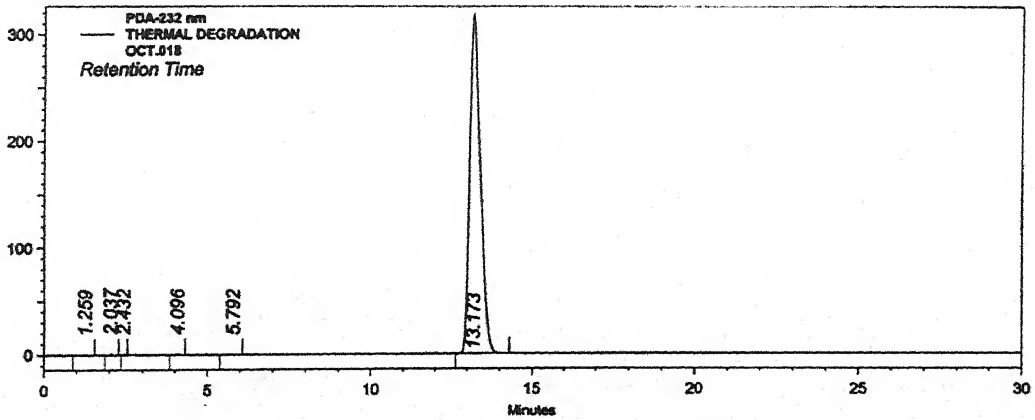
Acid degradation



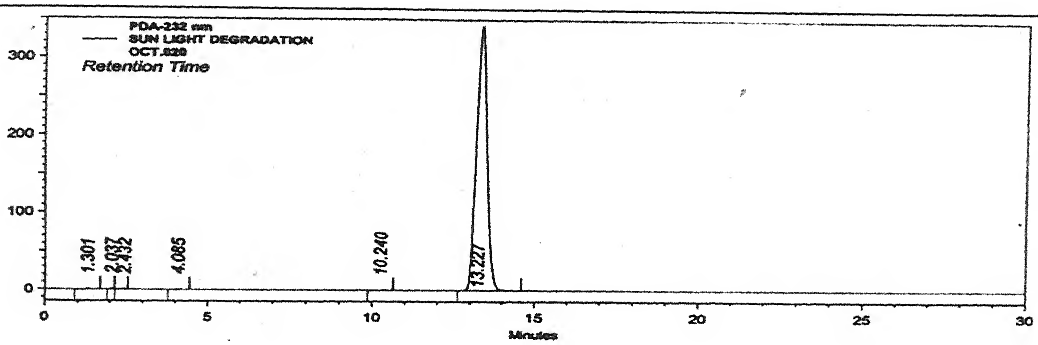
Alkali degradation



Peroxide degradation



Thermal degradation



Sun-light degradation

The result obtained from degradation study shows peak purity of Verapamil was 100 % as calculated by PDA detector, proving that no degradation product is interfering with the main peak. The % residual drug was calculated in comparison with the standard, which is 97.8, 80.9, 69.4, 99.3 and 100.6% for Acid, Alkali, Peroxide, Thermal and sunlight degradation respectively.

The result obtained during robustness shows that by changing deliberately, some internal and external parameters of the method does not influence the results. solution stability of 25 hrs was observed.

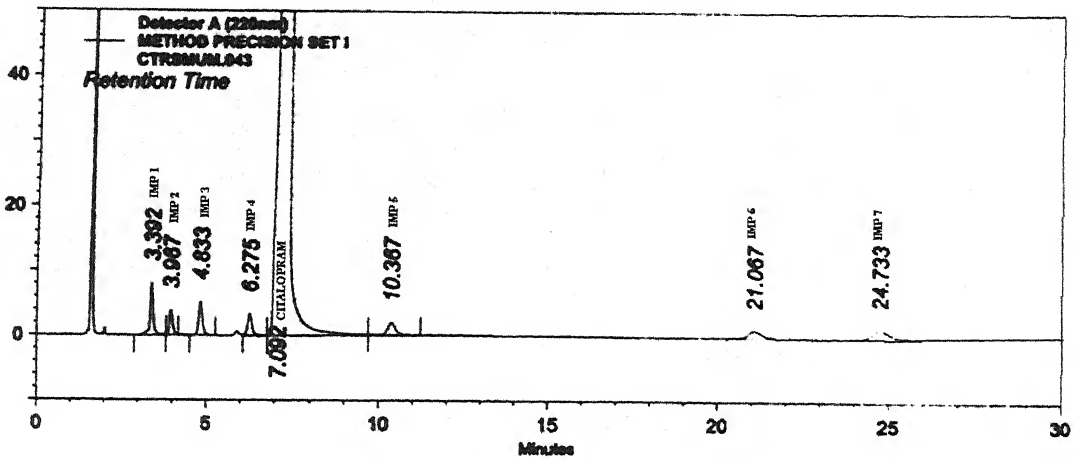
Table 5.5.1 : Summary of the performance parameters of the HPLC procedure for Verapamil Tablets

S.no	Parameters	Observed value
1.	System Suitability	
	a.Theoretical Plates	8155
	b. Tailing Factor	1.55
2.	Instrument Precision	RSD 0.27 %
3.	Method Precision	Label claim 103.3 %
4.	Linearity and range	Correlation coefficient(r^2) = 1.0000
5.	Accuracy	Mean recovery 100.3%
6.	Specificity	Peak Purity of Verapamil peak after degradation was 100.0 %
7.	Robustness	Difference from original condition 0.15%
8.	Solution stability	25 hrs

5.6 Citalopram

The retention time of Citalopram peak was about 7.1min.The system suitability is determined by obtaining the resolution factor between citalopram and impurity 5,which is found to be 10.2.Typical chromatograph of Citalopram (1000 ppm) along with spiked impurities at 0.2 % level,is shown in figure 5.6F1.

Figure 5.6F1 : Typical chromatograph of Citalopram



The relative retention time of all the impurites are summarized under table 5.6.1

Table 5.6.1 : The Relative retention time for Citalopram and its impurities

Components	Retention time(min)	Relative retention time
Citalopram	7.1	1.00
Impurity 1	3.4	0.48
Impurity 2	4.0	0.56
Impurity 3	4.8	0.68
Impurity 4	6.3	0.89
Impurity 5	10.4	1.60
Impurity 6	21.0	3.23
Impurity 7	24.7	3.80

The relative standard deviation of the area of the Citalopram and its impurities peak for replicate injections was found to be less than 5.0%.Table 5.6.2 shows detailed of instrument precision data.

Table 5.6.2 : Results for Instrument precision of Citalopram and its impurities

Injection	Detector response(area counts)							
	Imp 1	Imp 2	Imp 3	Imp 4	Imp 5	Imp 6	Imp 7	Citalopram
1	46180	22751	24072	28301	23606	28014	25014	15305
2	46057	22938	24057	28351	23374	27424	25558	15326
3	46148	22777	24096	28376	23506	27055	25253	15437
4	45891	22762	23967	28311	23593	27629	25450	15283
5	46031	22781	23958	28376	23545	28163	25254	15343
6	45950	22957	24035	28130	23441	27223	25892	15373
Mean	46043	22828	24031	28308	23511	27585	25404	15345
% RSD	0.24	0.41	0.24	0.33	0.38	1.59	1.20	0.36

Method precision shows a relative standard deviation of less than 5.0 % for all impurities, therefore the method can be said as precise. Details of method precision study is tabulated under table no. 5.6.3

Table 5.6.3 : Result of Method Precision for Citalopram impurities

Set #	Detector response(area counts)						
	Imp 1	Imp 2	Imp 3	Imp 4	Imp 5	Imp 6	Imp 7
1	0.1943	0.1942	0.1905	0.1944	0.2049	0.1968	0.1946
2	0.1928	0.1938	0.1956	0.1961	0.1978	0.1993	0.2050
3	0.1940	0.1968	0.2084	0.1981	0.2015	0.2082	0.2121
4	0.1929	0.1939	0.1989	0.1959	0.1985	0.1933	0.1960
5	0.1949	0.1976	0.2052	0.1976	0.2040	0.1898	0.2035
6	0.1943	0.1974	0.2033	0.1932	0.2035	0.2020	0.2002
Mean	0.1939	0.1956	0.2003	0.1959	0.2017	0.1982	0.2019
% RSD	0.43	0.94	3.30	0.95	1.48	3.29	3.19

The method was shown to be linear from 50 – 150 % of Citalopram limit concentration. A calibration curve was constructed using characteristic parameters for regression equation (Y= a + bx) and coefficient of correlation r^2 was found to be not less then 0.99.The details of linearity data is shown in table no. 5.6.4

Table 5.6.4 : Details for Citalopram and impurities of Linearity study

Set #	Impurities							
	Imp 1		Imp 2		Imp 3		Imp 4	
	Conc	Mean Area	Conc	Mean Area	Conc	Mean Area	Conc	Mean Area
1	1.14	27459	1.22	11253	1.20	11871	1.06	13800
2	1.71	37636	1.83	17182	1.80	17703	1.59	21380
3	2.28	45962	2.44	22555	2.40	23562	2.12	27981
4	2.85	55472	3.05	28076	3.00	29493	2.65	34871
5	3.42	66219	3.66	34172	3.60	35905	3.18	42280
Slope	16729.06		9300.55		9976.56		13292.64	
Intercept	8407.40		-45.93		-236.73		-118.33	
Correlation coefficient	0.9992		0.9998		0.9998		0.9998	

Set #	Impurities							
	Imp 5		Imp 6		Imp 7		CITALOPRAM	
	Conc	Mean Area	Conc	Mean Area	Conc	Mean Area	Conc	Mean Area
1	1.22	11361	1.22	11244	1.12	12037	0.50	8182
2	1.83	18333	1.83	18760	1.68	19295	0.76	12065
3	2.44	23496	2.44	27635	2.24	26124	1.01	15514
4	3.05	28839	3.05	31085	2.80	31885	1.26	18811
5	3.66	34955	3.66	40718	3.36	38309	1.51	23148
Slope	9458.14		11684.10		11631.19		14554.89	
Intercept	319.07		-2620.60		-523.93		872.80	
Correlation coefficient	0.9987		0.9929		0.9991		0.9990	

Figure 5.6F2 : Linearity graphs of Citalopram and impurities

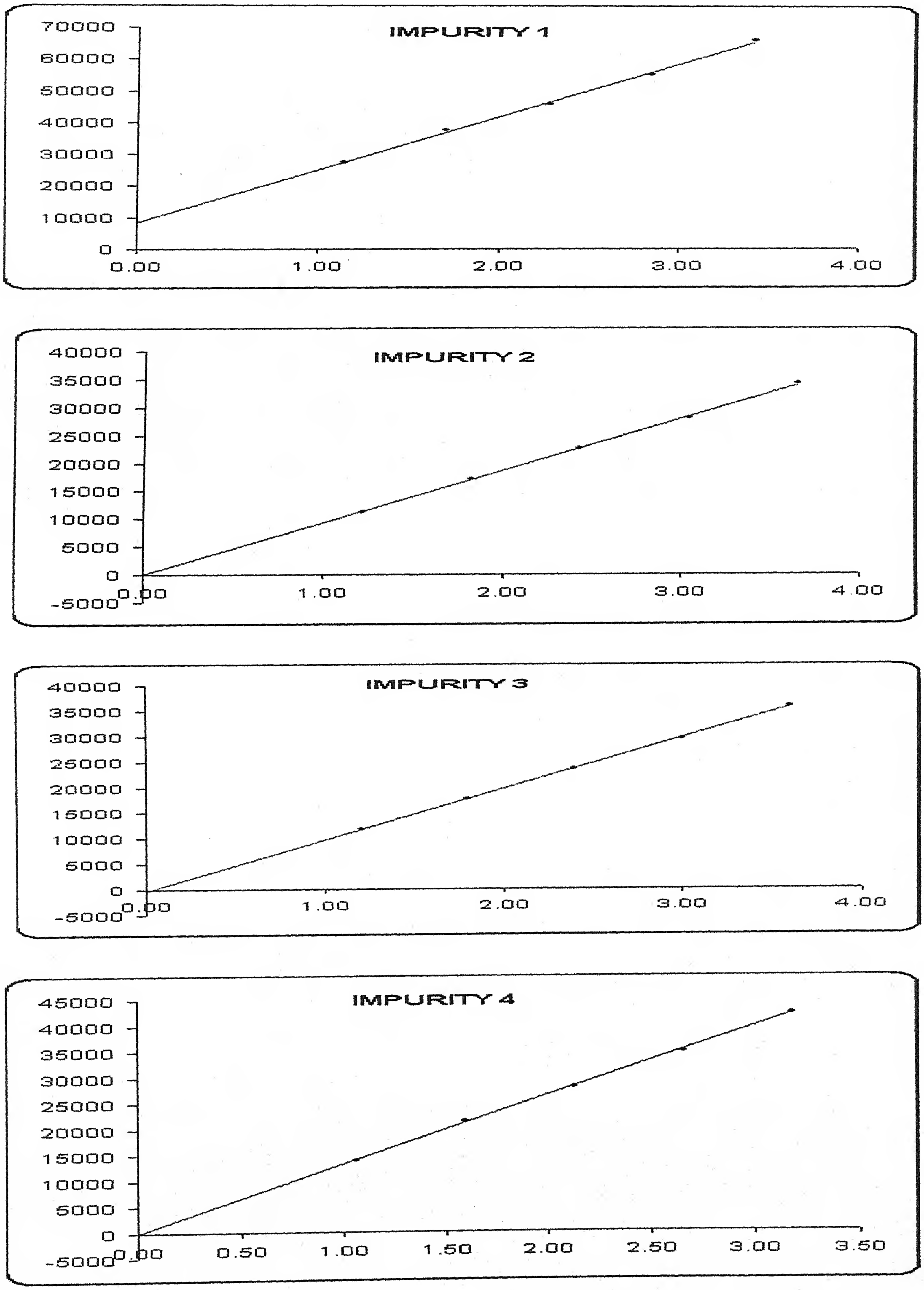
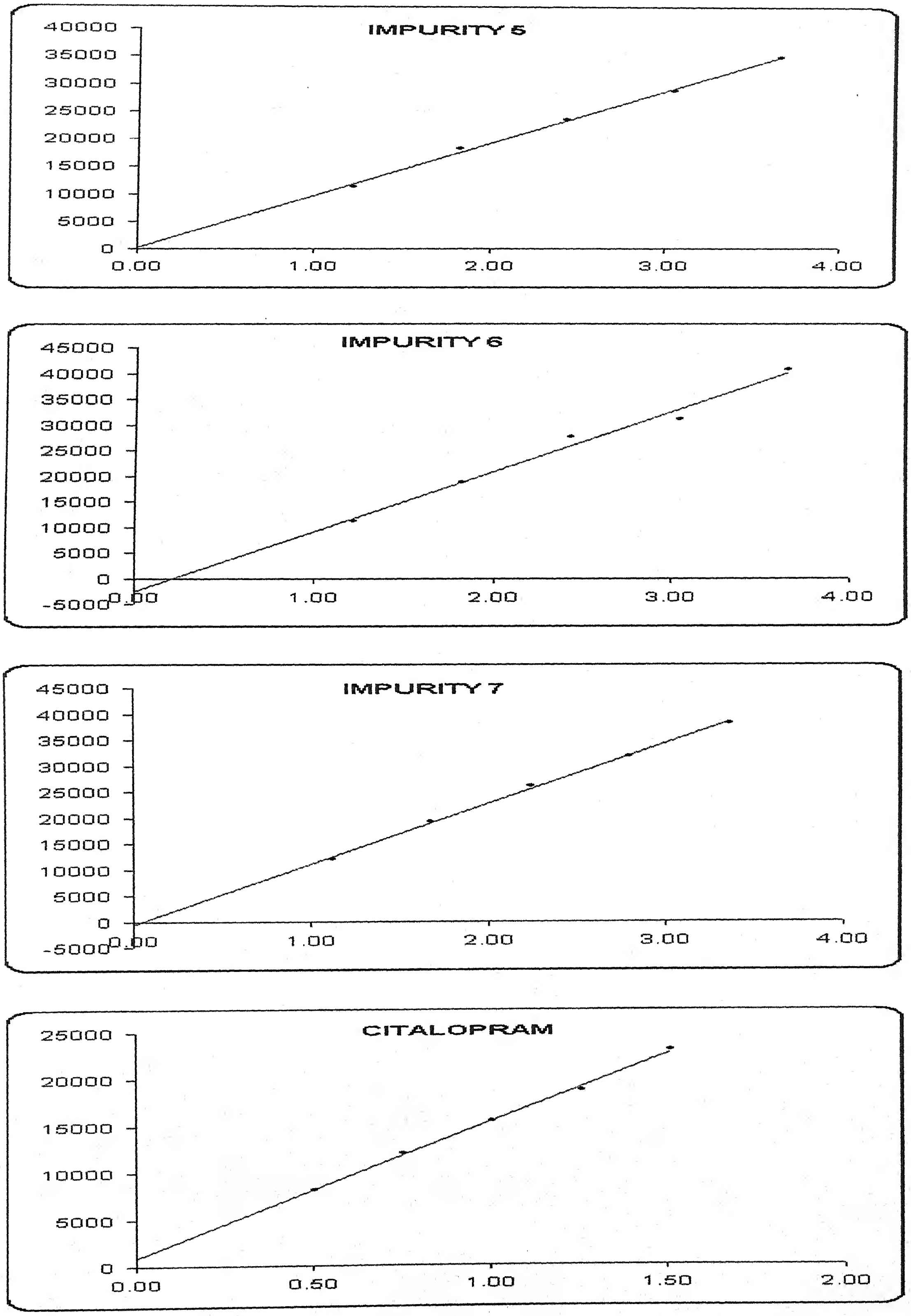


Figure 5.6F2 : Linearity graphs of Citalopram and impurities



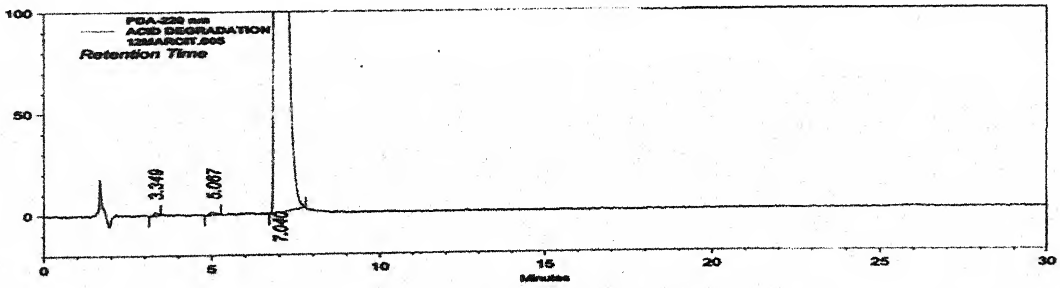
The mean recovery data for each level is within accepted values (95-105 % recovery) for 70,85,100,115 and 130 % of label claim, Therefore, these results indicated a good accuracy of the method for citalopram impurity profile. The details of recovery data is shown in table no. 5.6.5

Table 5.6.5 : Details for recovery study for Citalopram impurities

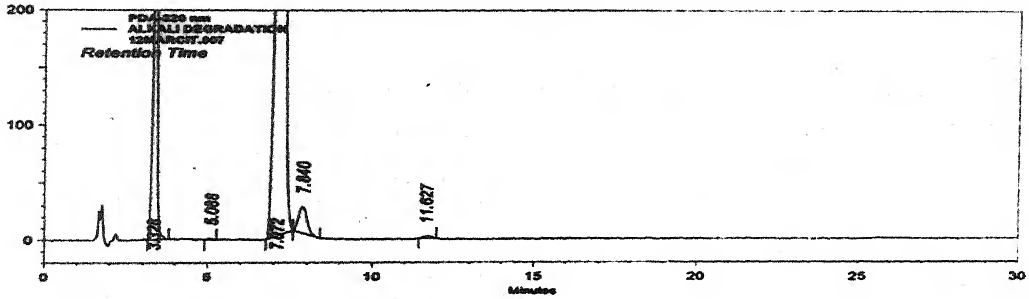
% level of standard	% Recovery						
	Imp 1	Imp 2	Imp 3	Imp 4	Imp 5	Imp 6	Imp 7
70	101.73	99.99	102.32	101.18	103.16	103.74	102.51
85	97.72	97.25	100.46	98.82	99.21	102.99	99.43
100	99.42	98.20	103.92	98.98	102.02	104.43	103.72
115	100.18	96.74	95.22	97.46	99.76	99.48	98.50
130	95.90	98.22	100.50	98.75	101.80	104.52	102.69
mean	98.99	98.08	100.49	99.03	101.19	103.03	101.37
% RSD	2.28	1.27	3.26	1.35	1.63	2.02	2.24

All the degradation peaks generated in the forced degradation studies were well separated and 3 point peak purity of Citalopram peak was always greater than 0.99. proving the stability indicating nature of the method. Major degradation was observed under Alkali and Peroxide degradation conditions. Typical chromatographs obtained in degradation study are shown in figure 5.6F3.

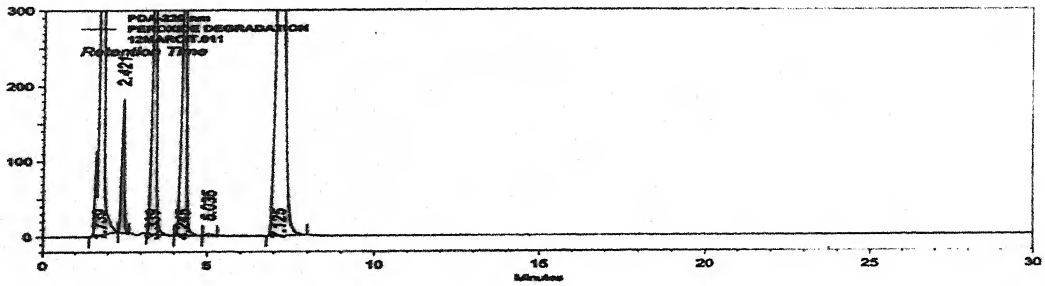
Figure 5.6F3 : Specificity study chromatographs of Citalopram



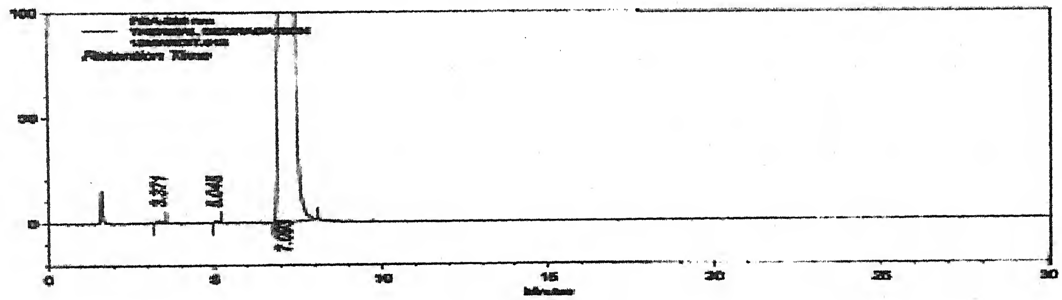
Acid degradation



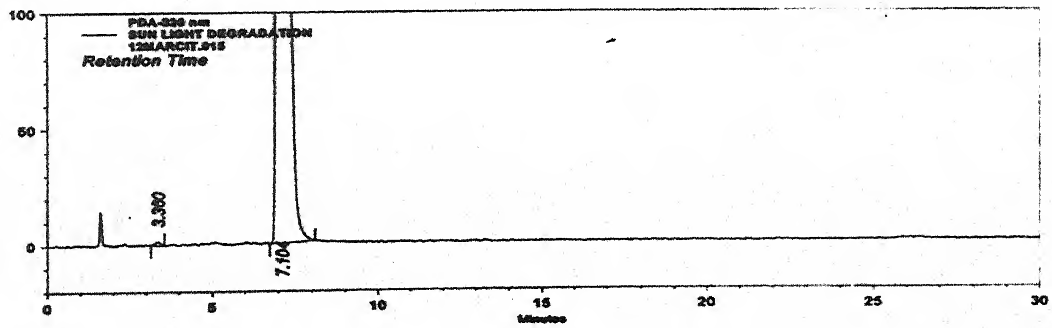
Alkali degradation



Peroxide degradation



Thermal degradation



Sun-light degradation

For determining the limit of detection (LOD) and limit of quantitation (LOQ), the method based on the residual standard deviation of a regression line and slope was adopted, a specific calibration curve was constructed .The results are tabulated below under table no. 5.6.6

Table 5.6.6 : Details for LOD and LOQ for Citalopram impurities

Components	Limit of quantitation		Limit of detection	
	(µg/ml)	(%)	(µg/ml)	(%)
Impurity 1	0.068	0.007	0.068	0.007
Impurity 2	0.073	0.007	0.073	0.007
Impurity 3	0.144	0.014	0.072	0.007
Impurity 4	0.127	0.013	0.062	0.006
Impurity 5	0.305	0.031	0.146	0.015
Impurity 6	0.610	0.061	0.305	0.031
Impurity 7	0.560	0.056	0.280	0.028
Citalopram	0.250	0.025	0.060	0.006

The result obtained during robustness shows that by changing deliberately, some internal and external parameters of the method does not influence the results. The results are tabulated below under table no. 5.6.7

Table 5.6.7 : Results for Robustness of method

Parameters	Imp 1	Imp 2	Imp 3	Imp 4	Imp 5	Imp 6	Imp 7
Original	0.231	0.214	0.335	0.210	0.238	0.232	0.332
Analyst change	0.229	0.212	0.348	0.211	0.234	0.238	0.340
Column Change	0.236	0.204	0.337	0.223	0.245	0.241	0.343
Column temp change	0.228	0.212	0.342	0.221	0.233	0.235	0.337
Mobile phase composition	0.232	0.214	0.348	0.210	0.236	0.241	0.330
Instrument change	0.244	0.213	0.351	0.196	0.235	0.244	0.337

Solution stability of 54 hrs was observed by periodically injecting stability solution. The details of peak area at different time intervals are tabulated under table 5.6.8. The result indicates that % deviation from mean initial area counts are not more than 5.0 % proving that solution is not needed to be freshly prepared.

Table 5.6.8 : Results for Solution stability of method

Time Hrs	Impurities							
	Imp 1		Imp 2		Imp 3		Imp 4	
	Mean Area counts	% deviation from mean initial area	Mean Area counts	% deviation from mean initial area	Mean Area counts	% deviation from mean initial area	Mean Area counts	% deviation from mean initial area
Initial	200724	0.00	113193	0.00	117915	0.00	139453	0.00
11	200997	0.14	113202	0.01	117934	0.02	139253	-0.14
18	200614	-0.05	112960	-0.21	117424	-0.42	138868	-0.42
30	200484	-0.12	113013	-0.16	117063	-0.72	138357	-0.79
47	201621	0.45	112801	-0.35	120163	1.91	138556	-0.64
54	201392	0.33	112895	-0.26	120221	1.96	138959	-0.35

Time Hrs	Impurities							
	Imp 5		Imp 6		Imp 7		Citalopram	
	Mean Area counts	% deviation from mean initial area	Mean Area counts	% deviation from mean initial area	Mean Area counts	% deviation from mean initial area	Mean Area counts	% deviation from mean initial area
Initial	117126	0.00	140745	0.00	129154	0.00	76015	0.00
11	116595	-0.45	139768	-0.69	129582	0.33	75740	-0.36
18	116228	-0.77	140576	-0.12	129112	-0.03	75711	-0.40
30	116592	-0.46	139694	-0.75	128038	-0.86	75318	-0.92
47	115959	-1.00	141534	0.56	127873	-0.99	75445	-0.75
54	116085	-0.89	138331	-1.72	129480	0.25	75663	-0.46

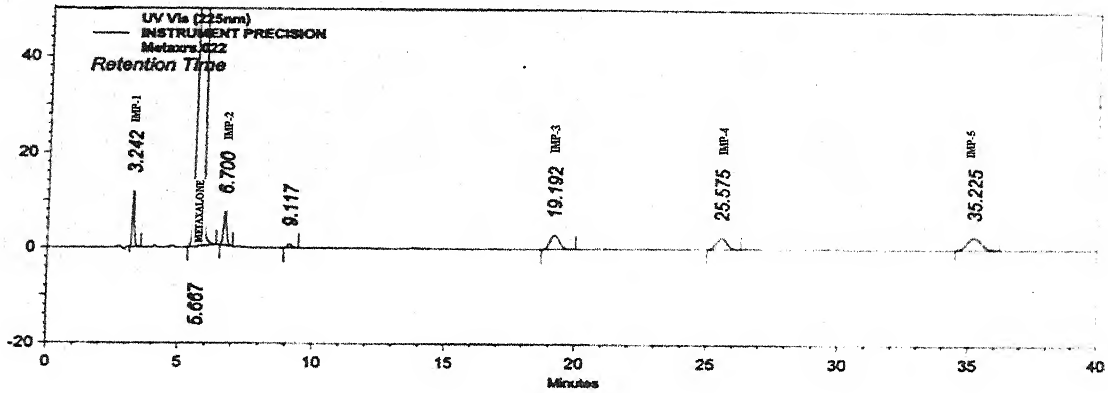
Table 5.6.9 : Summary of the performance parameters of the HPLC procedure for Citalopram bulk drug

Parameters	Observed Results							
System suitability Resolution	10.2							
	Imp 1	**Imp 2**	**Imp 3**	**Imp 4**	**Imp 5**	**Imp 6**	**Imp 7**	**Citalopram**
Instrument Precision % RSD Limit NMT 5.0 %	0.24	0.41	0.24	0.33	0.38	1.59	1.20	0.36
Method Precision mean	0.194	0.196	0.200	0.196	0.202	0.198	0.202	-
% RSD Limit NMT 5.0 %	0.43	0.94	3.30	0.95	1.48	3.29	3.19	-
Linearity and Range Coefficient of correlation Limit NLT 0.99	0.9992	0.9998	0.9998	0.9998	0.9987	0.9929	0.9991	0.9990
Accuracy % recovery Limit 95-105 %	98.99	98.08	100.49	99.03	101.19	103.03	101.37	-
Minimum quantitation level (%)	0.007	0.007	0.014	0.013	0.031	0.061	0.056	0.025
Minimum detection level (%)	0.007	0.007	0.007	0.006	0.015	0.031	0.028	0.006
Robustness	Difference NMT 10.0 % of impurity limit in original condition							
Original	0.231	0.214	0.335	0.210	0.238	0.232	0.332	-
Analyst	0.229	0.212	0.348	0.211	0.234	0.238	0.340	-
Column	0.236	0.204	0.337	0.223	0.245	0.241	0.343	-
Column temp	0.228	0.212	0.342	0.221	0.233	0.235	0.337	-
Mobile Phase composition	0.232	0.214	0.348	0.210	0.236	0.241	0.330	-
Instrument	0.244	0.213	0.351	0.196	0.235	0.244	0.337	-
Specificity	3 point peak purity not less than 0.99 in all degradation condition							
Solution stability	Upto 54 hrs							

5.7 Metaxalone

The retention time of Metaxalone peak was about 5.6 min.The system suitability is determined by obtaining the resolution factor between Metaxalone and impurity 4,which is found to be 4.8.Typical chromatograph of Metaxalone (1000 ppm) along with spiked impurities at 0.2 % level, is shown in figure 5.7F1.

Figure 5.7F1 : Typical chromatograph of Metaxalone



The relative retention time of all the impurites are summarized under table 5.7.1

Table 5.7.1 : The Relative retention time for Metaxalone and its impurities

Components	Retention time(min)	Relative retention time
Metaxalone	5.6	1.00
Impurity 1	3.2	0.6
Impurity 2	6.7	1.2
Impurity 3	19.2	3.4
Impurity 4	25.6	4.6
Impurity 5	35.2	6.3

The relative standard deviation of the area of the Metaxalone and its impurities peak for replicate injections was found to be less than 5.0%.Table 5.7.2 shows detailed of instrument precision data.

Table 5.7.2 : Results for Instrument precision of Metaxalone and its impurities

Injection	Detector response(area counts)					
	Imp 1	Imp 2	Imp 3	Imp 4	Imp 5	Metaxalone
1	66757	59436	71790	67104	100162	36034
2	66968	59385	71834	67157	100106	37199
3	66831	59520	71945	67077	100617	36647
4	66199	59204	72377	67389	100307	37280
5	66876	59531	72108	67100	99519	36845
6	66837	59584	71756	67408	100029	37066
Mean	66745	59443	71968	67206	100123	36845
% RSD	0.41	0.23	0.33	0.23	0.36	1.25

Method precision shows a relative standard deviation of less than 5.0 % for all impurities, therefore the method can be said as precise. Details of method precision study is tabulated under table no. 5.7.3

Table 5.7.3 : Result of Method Precision for Metaxalone impurities

Set #	Detector response(area counts)				
	Imp 1	Imp 2	Imp 3	Imp 4	Imp 5
1	0.2049	0.1993	0.1997	0.1992	0.2016
2	0.2052	0.1991	0.1997	0.1997	0.2020
3	0.2051	0.1996	0.1992	0.1994	0.2014
4	0.2052	0.1999	0.1999	0.2001	0.2025
5	0.2051	0.2008	0.1998	0.2000	0.2017
6	0.2053	0.2002	0.2001	0.1998	0.2018
Mean	0.2051	0.1998	0.1997	0.1997	0.2018
% RSD	0.07	0.31	0.14	0.17	0.18

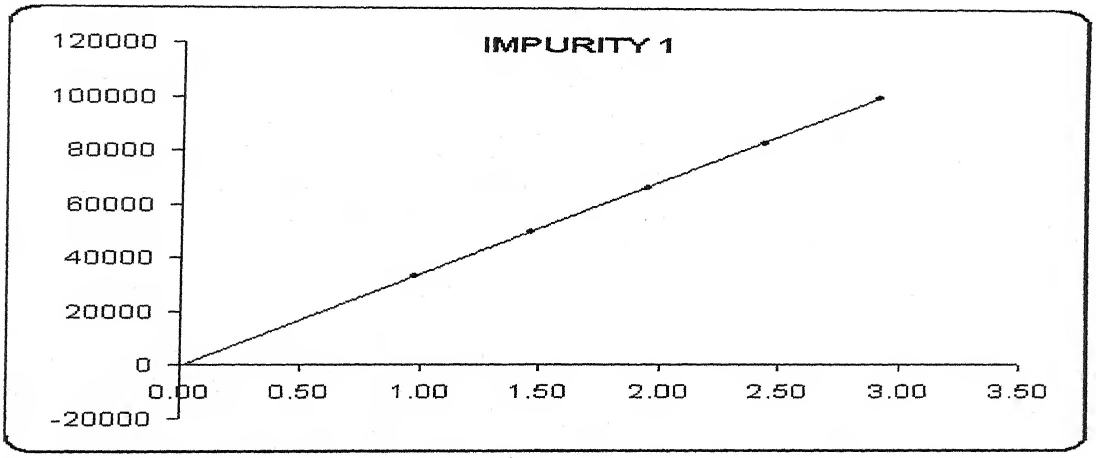
The method was shown to be linear from 50 – 150 % of Metaxalone limit concentration. A calibration curve was constructed using characteristic parameters for regression equation (Y= a + bx) and coefficient of correlation r^2 was found to be not less then 0.99.The details of linearity data is shown in table no. 5.7.4

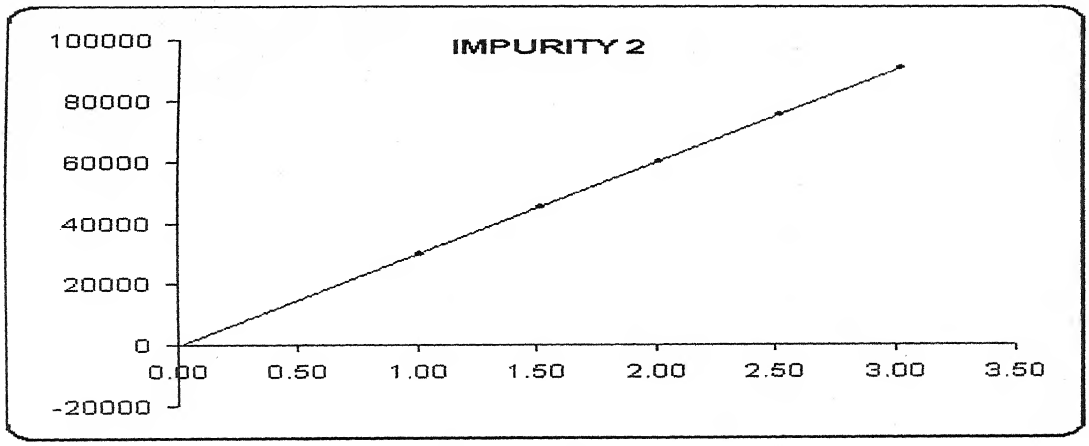
Table 5.7.4 : Details for Metaxalone and impurities of Linearity study

Set #	Impurities					
	Imp 1		Imp 2		Imp 3	
	Conc	Mean Area	Conc	Mean Area	Conc	Mean Area
1	0.97	33117	1.01	30122	1.04	36496
2	1.46	49797	1.51	45310	1.56	56057
3	1.94	66314	2.02	60314	2.08	74607
4	2.43	82827	2.52	75455	2.60	94021
5	2.92	99783	3.02	91052	3.12	112541
Slope	34230.86		30159.79		36548.72	
Intercept	-177.00		-351.53		-1276.93	
Correlation coefficient	1.000		1.000		1.000	

Set #	Impurities					
	Imp 4		Imp 5		METAXALONE	
	Conc	Mean Area	Conc	Mean Area	Conc	Mean Area
1	1.01	33325	1.04	50911	0.50	19701
2	1.52	50733	1.55	77972	0.74	29748
3	2.02	68044	2.07	101811	0.99	39818
4	2.53	85825	2.59	128534	1.24	49731
5	3.04	102878	3.11	154358	1.49	60402
Slope	34426.48		49702.25		40881.18	
Intercept	-1518.00		-265.87		-674.20	
Correlation coefficient	1.000		0.9999		0.9999	

Figure 5.7F2 : Linearity graphs of Metaxalone and impurities





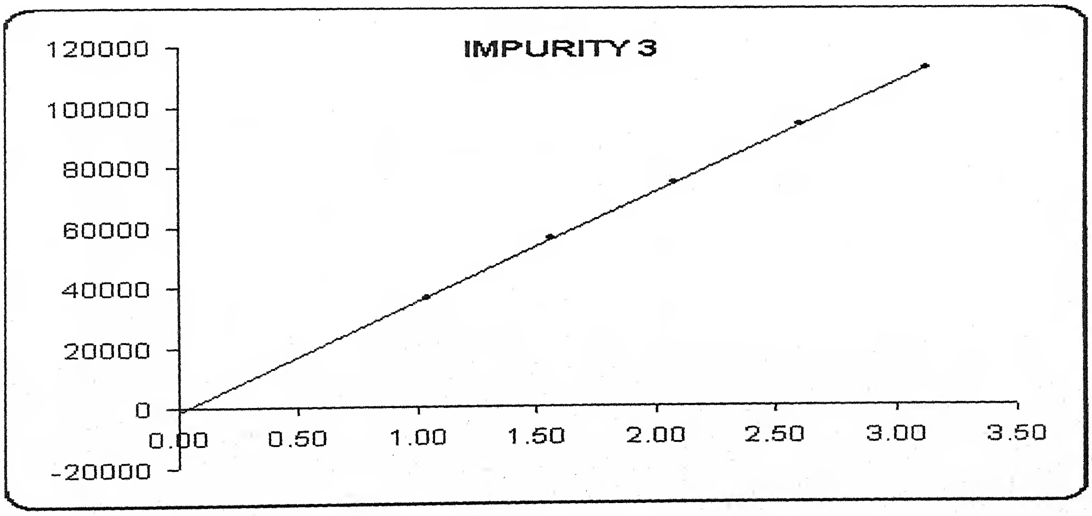
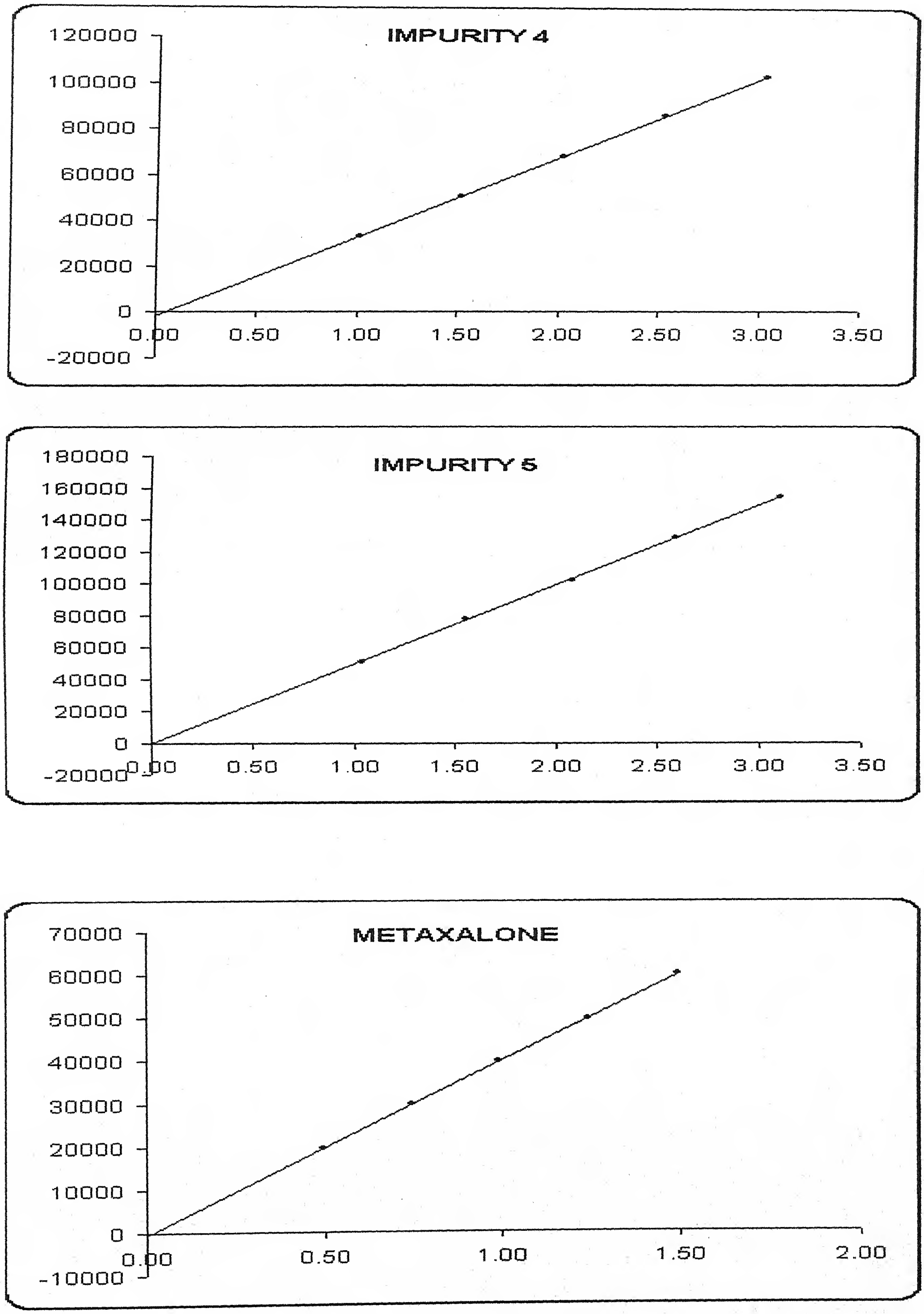


Figure 5.7F2 : Linearity graphs of Metaxalone and impurities



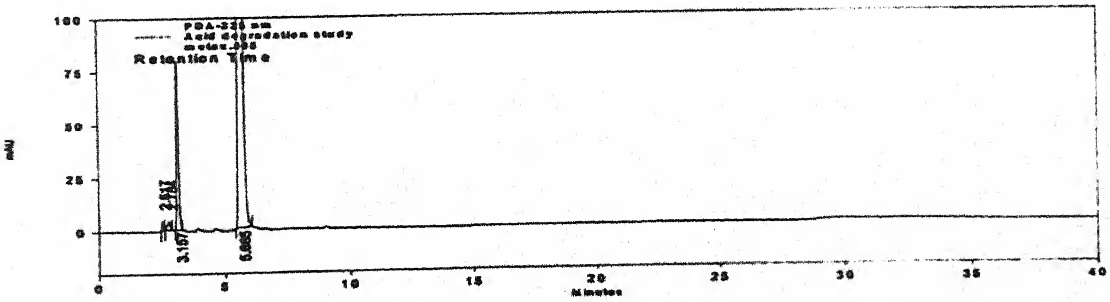
The mean recovery data for each level is within accepted values (95-105 % recovery) for 70, 85, 100,115 and 130 % of label claim, Therefore, these results indicated a good accuracy of the method for Metaxalone impurity profile. The details of recovery data is shown in table no. 5.7.5

Table 5.7.5 : Details for recovery study for Metaxalone impurities

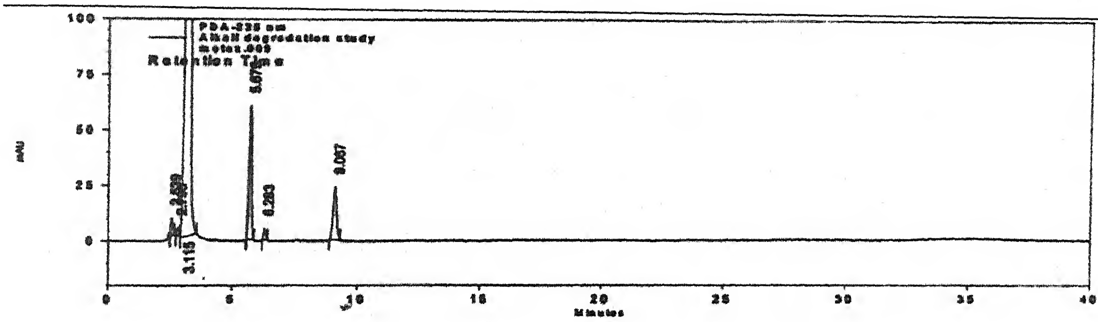
% level of standard	% Recovery				
	Imp 1	Imp 2	Imp 3	Imp 4	Imp 5
70	100.92	101.69	100.97	97.70	101.98
85	100.43	100.99	101.06	99.95	101.34
100	97.96	97.75	98.23	97.96	97.80
115	99.65	101.56	101.27	99.64	99.90
130	100.96	100.56	101.30	104.03	103.62
mean	99.98	100.51	100.57	99.85	100.93
% RSD	1.25	1.60	1.31	2.54	2.18

All the degradation peaks generated in the forced degradation studies were well separated and 3 point peak purity of Metaxalone peak was always greater than 0.99. proving the stability indicating nature of the method. Major degradation was observed under Alkali and Peroxide degradation conditions. Typical chromatographs obtained in degradation study are shown in figure 5.7F3.

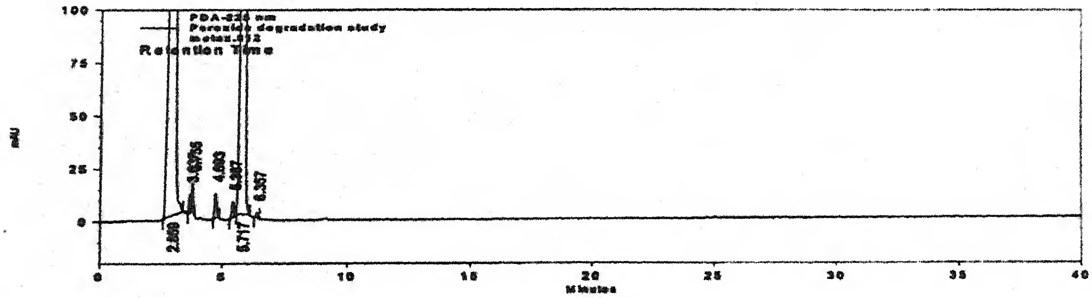
Figure 5.7F3 : Specificity study chromatographs of Metaxalone



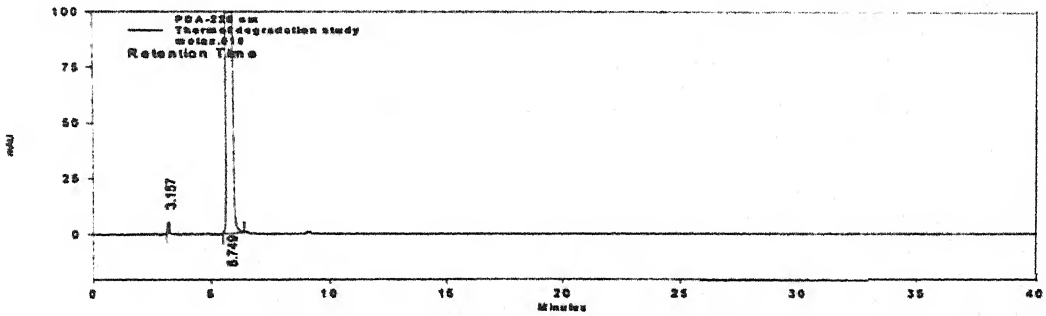
Acid degradation



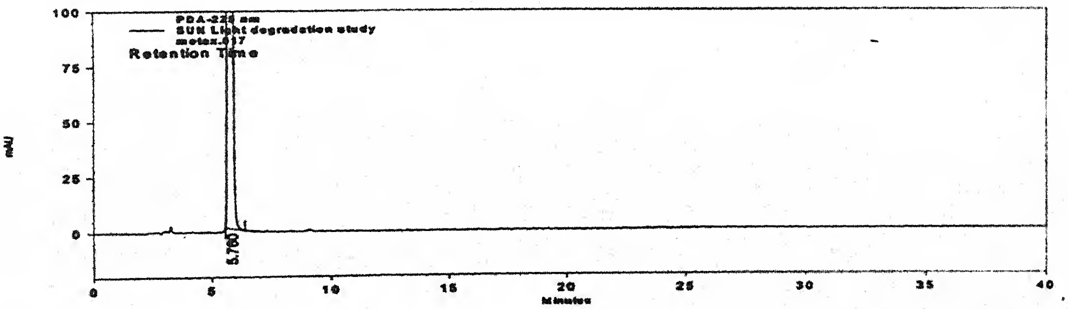
Alkali degradation



Peroxide degradation



Thermal degradation



Sun-light degradation

For determining the limit of detection (LOD) and limit of quantitation (LOQ), the method based on the residual standard deviation of a regression line and slope was adopted, a specific calibration curve was constructed .The results are tabulated below under table no. 5.7.6

Table 5.7.6 : Details for LOD and LOQ for Metaxalone impurities

Components	Limit of quantitation		Limit of detection	
	(µg/ml)	(%)	(µg/ml)	(%)
Impurity 1	0.115	0.012	0.029	0.003
Impurity 2	0.118	0.012	0.029	0.003
Impurity 3	0.120	0.012	0.030	0.003
Impurity 4	0.245	0.025	0.029	0.003
Impurity 5	0.250	0.025	0.030	0.003
Metaxalone	0.059	0.006	0.015	0.002

The result obtained during robustness shows that by changing deliberately, some internal and external parameters of the method does not influence the results. The results are tabulated below under table no. 5.7.7

Table 5.7.7 : Results for Robustness of method

Parameters	Imp 1	Imp 2	Imp 3	Imp 4	Imp 5
Original	0.2049	0.1993	0.1997	0.1992	0.2016
Analyst change	0.2012	0.2009	0.2006	0.1994	0.2010
Column Change	0.2038	0.1996	0.2011	0.2006	0.2015
Column temp change	0.2023	0.1964	0.2078	0.2073	0.2103
Mobile phase composition	0.1993	0.2003	0.2007	0.2004	0.2011
Instrument change	0.2002	0.2014	0.2031	0.1986	0.2016

Solution stability of 43 hrs was observed by periodically injecting stability solution. The details of peak area at different time intervals are tabulated under table 5.7.8. The result indicates that % deviation from mean initial area counts are not more than 5.0 % proving that solution is not needed to be freshly prepared.

Table 5.7.8 : Results for Solution stability of method

Time Hrs	Impurities					
	Imp 1		Imp 2		Imp 3	
	Mean Area counts	% deviation from mean initial area	Mean Area counts	% deviation from mean initial area	Mean Area counts	% deviation from mean initial area
Initial	810479	0.00	734701	0.00	907498	0.00
4	816131	-0.70	738899	-0.57	914974	-0.82
11	816358	-0.73	739356	-0.63	914170	-0.74
17	807582	0.36	733996	0.10	905236	0.25
43	806728	0.46	733413	0.18	903432	0.45

Time Hrs	Impurities					
	Imp 4		Imp 5		METAXALONE	
	Mean Area counts	% deviation from mean initial area	Mean Area counts	% deviation from mean initial area	Mean Area counts	% deviation from mean initial area
Initial	841136	0.00	1246782	0.00	456531	0.00
4	845986	-0.58	1255771	-0.72	459604	-0.67
11	844529	-0.40	1256237	-0.76	459453	-0.64
17	836756	0.52	1247703	-0.007	455775	0.17
43	834961	0.73	1249581	-0.22	455338	0.26

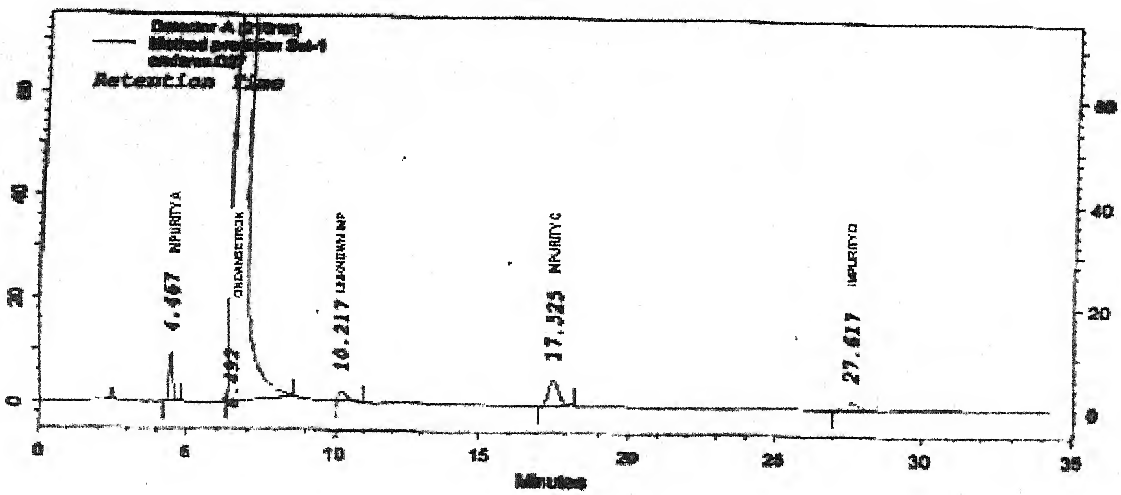
Table 5.7.9 : Summary of the performance parameters of the HPLC procedure for Metaxalone bulk drug

Parameters	Observed Results					
System suitability Resolution	4.8					
	Imp 1	**Imp 2**	**Imp 3**	**Imp 4**	**Imp 5**	**Metaxalone**
Instrument Precision % RSD Limit NMT 5.0 %	0.41	0.23	0.33	0.23	0.36	1.25
Method Precision mean	0.2051	0.1998	0.1997	0.1997	0.2018	-
% RSD Limit NMT 5.0 %	0.07	0.31	0.14	0.17	0.18	-
Linearity and Range Coefficient of correlation Limit NLT 0.99	1.0000	1.0000	1.0000	1.0000	0.9999	0.9999
Accuracy % recovery Limit 95-105 %	99.98	100.51	100.57	99.85	100.93	-
Minimum quantitation level (%)	0.012	0.012	0.012	0.025	0.025	0.006
Minimum detection level (%)	0.003	0.003	0.003	0.003	0.003	0.002
Robustness	Difference NMT 10.0 % of impurity limit in original condition					
Original	0.2049	0.1993	0.1997	0.1992	0.2016	-
Analyst	0.2012	0.2009	0.2006	0.1994	0.2010	-
Column	0.2038	0.1996	0.2011	0.2006	0.2015	-
Column temp	0.2023	0.1964	0.2078	0.2073	0.2103	-
Mobile Phase composition	0.1993	0.2003	0.2007	0.2004	0.2011	-
Instrument	0.2002	0.2014	0.2031	0.1986	0.2016	-
Specificity	3 point peak purity not less than 0.99 in all degradation condition					
Solution stability	Upto 43 hrs					

5.8 Ondansetron

The retention time of Ondansetron peak was about 6.5 min. The system suitability is determined by obtaining the resolution factor between Ondansetron and impurity A, which is found to be 7.5.Typical chromatograph of Ondansetron (500 ppm) along with spiked impurities at 0.2 % level A, C and 0.1 % of impurity D is shown in figure 5.8F1.

Figure 5.8F1 : Typical chromatograph of Ondansetron



The relative retention time of all the impurites are summarized under table 5.8.1

Table 5.8.1 : The Relative retention time for Ondansetron and its impurities

Components	Retention time(min)	Relative retention time
Ondansetron	6.5	1.00
Impurity A	4.5	0.7
Impurity C	17.5	2.7
Impurity D	27.6	4.2

The relative standard deviation of the area of the Ondansetron and its impurities peak for replicate injections was found to be less than 5.0%.Table 5.8.2 shows detailed of instrument precision data.

Table 5.8.2 : Results for Instrument precision of Ondansetron and its impurities

Injection	Detector response(area counts)			
	Imp A	Imp C	Imp D	Ondansetron
1	82585	109090	53164	23500366
2	81873	109454	52686	23625433
3	81663	107484	55231	23352975
4	82332	109518	55572	23430542
5	81297	111916	55982	23356647
6	81837	110901	55972	23172295
Mean	81931	109727	54768	23406376
% RSD	0.57	1.40	2.67	0.66

Method precision shows a relative standard deviation of less than 5.0 % for all impurities, therefore the method can be said as precise. Details of method precision study is tabulated under table no. 5.8.3

Table 5.8.3 : Result of Method Precision for Ondansetron impurities

Set #	Detector response(area counts)		
	Imp A	Imp C	Imp D
1	0.1997	0.1960	0.0988
2	0.1982	0.1968	0.0991
3	0.1968	0.1918	0.0937
4	0.1987	0.1886	0.0970
5	0.2003	0.2011	0.1033
6	0.2004	0.2042	0.1039
Mean	0.1990	0.1964	0.0993
% RSD	0.70	2.92	3.88

The method was shown to be linear from 50 – 150 % of Ondansetron limit concentration. A calibration curve was constructed using characteristic parameters

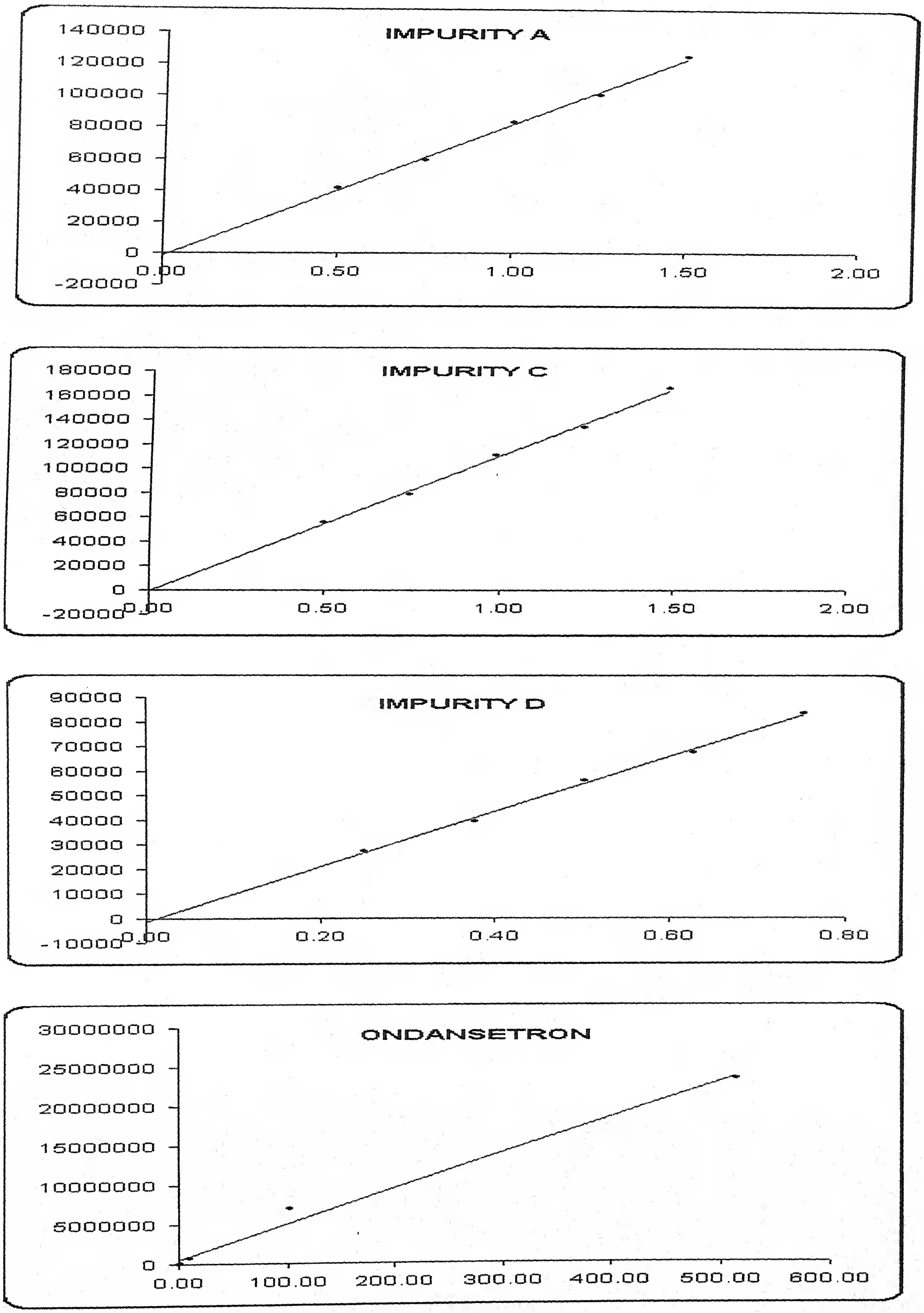
for regression equation (Y= a + bx) and coefficient of correlation r^2 was found to be not less then 0.99.The details of linearity data is shown in table no. 5.8.4

Table 5.8.4 : Details for Ondansetron and impurities of Linearity study

Set #	Impurities			
	Imp A		Imp C	
	Conc	Mean Area	Conc	Mean Area
1	0.50	41470	0.50	55702
2	0.75	59049	0.75	78234
3	1.00	83335	0.99	110645
4	1.25	100500	1.24	133370
5	1.50	125392	1.49	165502
Slope	83650.81		110513.01	
Intercept	-1768.47		-1203.60	
Correlation coefficient	0.9982		0.9982	

Set #	Impurities			
	Imp D		ONDANSETRON	
	Conc	Mean Area	Conc	Mean Area
1	0.25	27459	0.26	25383
2	0.38	39660	1.03	70711
3	0.50	56104	10.30	724598
4	0.63	67805	103.00	6976125
5	0.75	83715	515.00	23533798
Slope	112077.82		45259.25	
Intercept	-1314.20		567191.13	
Correlation coefficient	0.9987		0.9952	

Figure 5.8F2 : Linearity graphs of Ondansetron and impurities



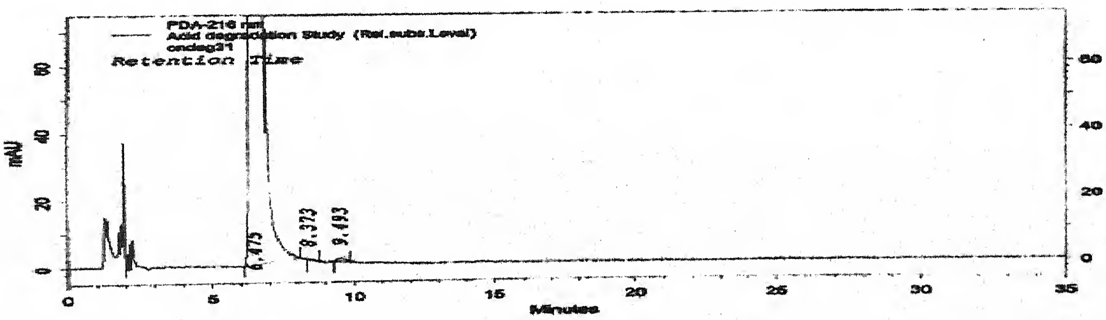
The mean recovery data for each level is within accepted values (95-105 % recovery) for 70, 85,100,115 and 130 % of label claim, Therefore, these results indicated a good accuracy of the method for Ondansetron impurity profile. The details of recovery data is shown in table no. 5.8.5

Table 5.8.5 : Details for recovery study for Ondansetron impurities

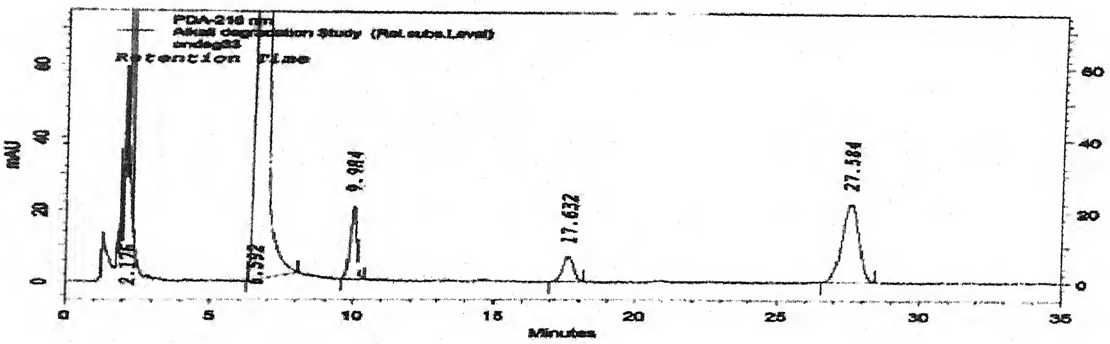
% level of STD	% Recovery		
	Imp A	Imp C	Imp D
70	99.89	102.19	101.75
85	100.23	100.58	98.10
100	98.13	98.17	99.24
115	99.14	97.48	99.37
130	100.75	100.41	98.75
mean	99.63	99.77	99.44
% RSD	1.02	1.92	1.39

All the degradation peaks generated in the forced degradation studies were well separated and 3 point peak purity of Ondansetron peak was always greater than 0.99. proving the stability indicating nature of the method. Major degradation was observed under Alkali and Peroxide degradation conditions. Typical chromatographs obtained in degradation study are shown in figure 5.8F3.

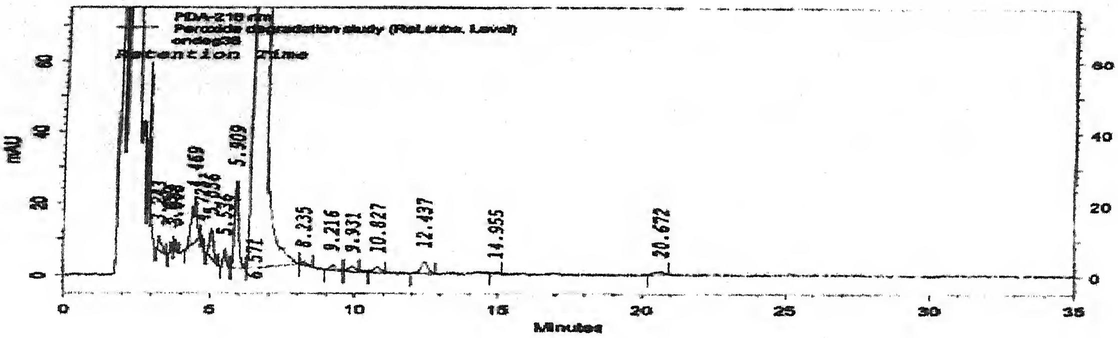
Figure 5.8F3 : Specificity study chromatographs of Ondansetron



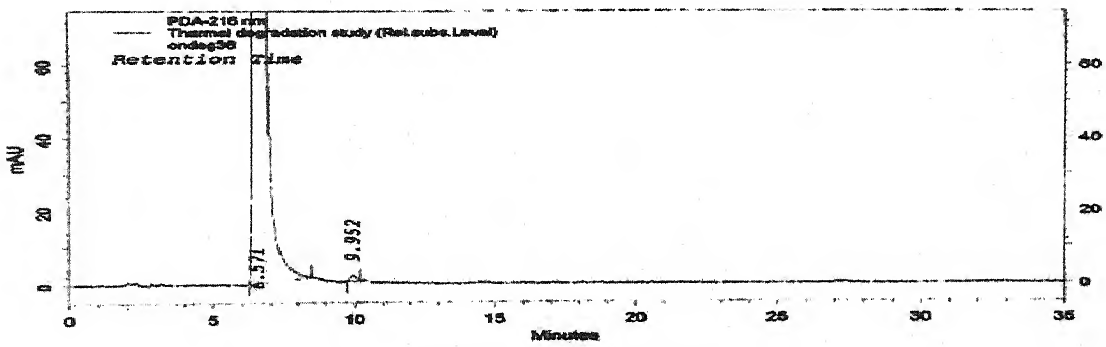
Acid degradation



Alkali degradation



Peroxide degradation



Thermal degradation

For determining the limit of detection (LOD) and limit of quantitation (LOQ), the method based on the residual standard deviation of a regression line and slope was adopted, a specific calibration curve was constructed .The results are tabulated below under table no. 5.8.6

Table 5.8.6 : Details for LOD and LOQ for Ondansetron impurities

Components	Limit of quantitation		Limit of detection	
	(µg/ml)	(%)	(µg/ml)	(%)
Impurity A	0.125	0.030	0.030	0.006
Impurity C	0.124	0.030	0.030	0.006
Impurity D	0.126	0.030	0.030	0.006

The result obtained during robustness shows that by changing deliberately, some internal and external parameters of the method does not influence the results. The results are tabulated below under table no. 5.8.7

Table 5.8.7 : Results for Robustness of method

Parameters	Imp A	Imp C	Imp D
Original	0.196	0.195	0.093
Analyst change	0.197	0.193	0.098
Column Change	0.202	0.210	1.00
Flow rate change	0.198	0.200	0.097
Mobile phase composition	0.210	0.197	0.100
Instrument change	0.203	0.199	0.097

Solution stability of 43 hrs was observed by periodically injecting stability solution. The details of peak area at different time intervals are tabulated under table 5.8.8. The result indicates that % deviation from mean initial area counts are not more than 5.0 % proving that solution is not needed to be freshly prepared.

Table 5.8.8 : Results for Solution stability of method

Time Hrs	Impurities			
	Imp A		Imp C	
	Mean Area counts	% deviation from mean initial area	Mean Area counts	% deviation from mean initial area
Initial	7537840	-	10338525	-
4	7450931	1.15	10261906	0.74
30	7431283	1.41	10323326	0.15

Time Hrs	Impurities			
	Imp D		Ondansetron	
	Mean Area counts	% deviation from mean initial area	Mean Area counts	% deviation from mean initial area
Initial	10403485	-	6474547	-
4	10283815	1.15	6433252	0.64
30	10696541	-2.82	6471746	0.04

Table 5.8.9 : Summary of the performance parameters of the HPLC procedure for Ondansetron bulk drug

Parameters	Observed Results			
System suitability Resolution	4.8			
	Imp A	**Imp C**	**Imp D**	**Ondansetron**
Instrument Precision % RSD Limit NMT 5.0 %	0.57	1.40	2.67	0.66
Method Precision mean	0.1990	0.1964	0.0993	-
% RSD Limit NMT 5.0 %	0.70	2.92	3.88	-
Linearity and Range Coefficient of correlation Limit NLT 0.99	0.9982	0.9982	0.9987	0.9952
Accuracy % recovery Limit 95-105 %	99.63	99.77	99.44	-
Minimum quantitation level (%)	0.03	0.03	0.03	-
Minimum detection level (%)	0.006	0.006	0.006	-
Robustness	Difference NMT 10.0 % of impurity limit in original condition			
Original	0.196	0.195	0.093	-
Analyst	0.197	0.193	0.098	-
Column	0.202	0.210	1.00	-
Flow rate	0.198	0.200	0.097	-
Mobile Phase composition	0.210	0.197	0.100	-
Instrument	0.203	0.199	0.097	-
Specificity	3 point peak purity not less than 0.99 in all degradation condition			
Solution stability	Upto 30 hrs			

CHAPTER – 6

SUMMARY

Modern medicines for human use are required to comply with specific standards and regulations set forth by the concerned authorities. The efficacy and safety of medicinal products can only be assured by analytical monitoring of its quality. Hence, the quality control laboratory forms the heart of drug industry. It is here that various procedures are required for analysis of drug formulations.

Modern scientists have currently available to them, an amazing array of powerful analytical tools for obtaining qualitative and quantitative data about the properties and composition of matter. Pharmaceutical analysis is indispensable for the pharmaceutical industry. It comprises of various methods and techniques dealing with the analysis of drug and other materials used in pharmaceutical preparations. In manufacturing laboratories, pharmacists often have to perform physical, chemical and biological analysis, either in the course of developing dosage forms of new drugs or in the control of quality of products. The pharmaceutical analyst, therefore must be sufficiently well versed in analytical procedures not only to apply known techniques but also to devise new better techniques wherever necessary.

The field of pharmaceutical science is expanding rapidly with the invention of new drugs and novel drug delivery systems. Depending upon requirement of different patient class, different kind of dosage forms such as tablets, capsules, injections, syrups, suspensions, sustained release tablets etc. are designed. Over

the past two decades various regulatory bodies such as USFDA (United States Food and Drug Administration), WHO (World Health Organisation), British Pharmacopoeial Commission have taken very serious view about the quality of bulk drugs and pharmaceuticals which are manufactured. These agencies in consultation and collaboration with various research organizations and industries have been monitoring the efficacy of various drugs and their generic equivalent. Based on these studies some of the parameters , which are introduced, as mandatory requirements for different pharmaceutical products are: type of polymorph, chiral purity of the active molecule and dissolution rate of the dosage form. Considering the importance of pharmacological effect or adverse reactions of these drugs and their metabolites/degradation products/related substances, efforts have been initiated and are on to achieve consistent quality by issuing various guidelines on manufacturing and quality control of these products. Due to these stringent regulatory as well as ethical requirements, it becomes equally important to monitor the quality of these drug substances with respect to their purity and dosage forms at the time of release and at various stages of its shelf life. Quality control and quality assurance functions today have greater significance than ever. There are various analytical techniques, which have been used to monitor the quality of the pharmaceutical products. Looking at various validation criterions an analytical method needs to meet, in today's stringent quality requirements, chromatographic techniques are the method of choice in most of the cases. High pressure liquid chromatography (HPLC) has been considered as the most versatile technique because of availability of different types of stationary phases, unlimited choice of mobile phases, varieties of detectors to be chosen from a range and as a whole applicability to a wide range of compounds

which are not possible to analyse using other classical analytical techniques.

It was therefore thought worth to develop new methods of analysis for newly developed pharmaceutically important formulations using the HPLC technique. Stability indicating HPLC methods for various categories of drug formulations (which are required in day to day life) such as Antihypertensive, Anticonvulsant, Antiemetic and Muscle relaxant have been developed.

Short summary of all these developed methods is given below.

1. Candesartan tablets

The mobile phase consisted of 6.8 g KH_2PO_4 /1000ml water (buffer)and Methanol (40:60).Chromatography was performed on Thermohypersil C18, (250 x 4.6 mm, 5µm) column at a flow rate of 1.0 ml/min. The drug along with its degraded products was detected at 220 nm using PDA detector.

2. Captopril tablets

A mixture of water : acetonitrile: tetrahydrofuran :methane sulfonic acid in the ratio of 80:10:10:0.1was used as mobile phase. Chromatography was performed on Phenomenex, Luna C8, (250 x 4.6 mm, 5µm) column at a flow rate of 1.0 ml/min. The drug along with its degraded products was detected at 220 nm using PDA detector.

3. Propranolol tablets

A mixture of 5.0 g Triethylamine /1000ml water pH 4.0 ± 0.1 by HCOOH): acetonitrile::70:30. was used as mobile phase. Chromatography was performed on Luna C18, (250 x 4.6 mm, 5µm) column at a flow rate of 1.5 ml/min. The drug was detected at 225 nm along with its degradation products.

4. Terazosin tablets

The mobile phase consisted of Buffer(6.8 g KH_2PO_4 /1000ml) and Methanol. in the ratio of 60:40. Chromatography was performed on Thermohypersil,C18,250 * 4.6 mm,5 µm column at a flow rate of 1.0 ml/min. The detector was set at 245 nm and all the degradants were well separated.

5. Verapamil tablets

A mixture of (1.4 g Na_2HPO_4 /1000ml, pH adjusted to 7.0 ± 0.1 by H_3PO_4) and Acetonitrile in the ratio of 50:50.was used as mobile phase. Chromatography was performed on Thermohypersil C18, (250 x 4.6 mm, 5µm) column at a flow rate of 2.0 ml/min. The drugs along with their degraded products were detected at 232nm using PDA detector.

6. Citalopram Bulk drug

A mixture of buffer (1.3 gm diammonium hydrogen orthophosphate in 1000ml water + 2ml of triethylamine, pH 6.8 ± 0.1 by orthophosphoric acid) and Methanol, Acetonitrile in the ratio of 45: 45:10. .0.94g of sodium hexane sulphonic acid.was added ,then filtered and degassed and used as mobile phase. Chromatography was performed on Restek C18, 250*4.6mm, 5µ at a flow rate of 2.0 ml/min. column temperature was kept at 40°C.The drug alongwith its degraded products was detected at 220 nm.

7. Metaxalone Bulk drug

The mobile phase consisted of aqueous buffer (6.0 gm KH_2PO_4in 1000ml water pH 3.0 ± 0.1 by H_3PO_4) and Acetonitrile in the ratio of 50:50 Chromatography was performed at a flow rate of 1.0 ml/min on Hypersil C8, BDS, 250*4.6mm, 5μ column. The detector was set at 225 nm.

8. Ondansetron Bulk drug

The mixture of buffer (2.7 gm KH_2PO_4in 1000ml water + 5.0 ml of triethyl amine pH 3.0 ± 0.1 by H_3PO_4) and Acetonitrile in the ratio of 70:30 was used as mobile phase. Chromatography was performed at a flow rate of 1.2 ml/min. on Luna,C8,250*4.6mm, 5μ column The detector was set at 216 nm.

In all, five methods have been developed and validated for Five different formulations and three methods for three different bulk drugs. All these methods were found to be simple, rapid, reproducible, stability indicating and capable of assaying the drugs accurately from formulations in presence of excipients and their degradation products, Where as bulk drug method provides method for impurity profiling of the drug. The statistical parameters determined for the estimation of each drug in a formulation and bulk drug were found **to be satisfactory. All these methods were found to be robust under variety of test conditions.** All these methods can be utilised successfully for the analysis of preformulation studies, in-process analysis, routine quality control analysis, Stability studies of bulk and formulation products.

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